Sunday or Federal holiday, i.e. Monday, January 7, 2002, is considered timely under 37 C.F.R. §1.7. Accordingly, this Amendment is being timely filed.

Please amend the subject application as follows:

In the claims:

Please cancel claims 30-33 without prejudice or disclaimer to applicants' right to pursue the subject matter of these claims in a later-filed application.

REMARKS

Claims 30-33 and 41-58 are pending in the subject application. Applicants have hereinabove canceled claims 30-33 without prejudice or disclaimer to applicants right to pursue the subject matter of these claims in a later-filed application. This amendment does not involve any issue of new matter. Therefore, entry of this amendment is respectfully requested such that claims 41-58 will be pending.

Objection to the specifications

Figures

The Examiner objected to the specification because of the following alleged informalities: The Examiner stated that the drawings are objected to for reasons illustrated in Form PTO 948. The Examiner stated that corrections will be required in the event any claims are allowable.

In response, applicants will provide substitute drawings upon the indication of allowable subject matter.

Objection to claims 30, 45 and 55

The Examiner objected to claim 30 because it depends from claim 27, which was canceled. The Examiner objected to claims 45 and 55 because they allegedly recite or encompass non-elected inventions. The Examiner stated that appropriate correction is required.

In response, without conceding the correctness of the Examiner's position, but to expedite prosecution of the subject application, applicants have herein canceled claim 30 without prejudice or disclaimer to applicants right to pursue the subject matter of these claims in a later-filed application. Applicants respectfully point out that 37 C.F.R. §1.141 provides that a reasonable number of species may still be claimed in one application provided the application also includes an allowable claim generic to all the claimed species and all the claims to species in excess of one are written in dependent form. Applicants contend that claims 45 and 55 contain a reasonable number of claimed species which are written in dependent form for a genus claim, e.g. claim 44 and claim 47 respectively. Accordingly, applicants respectfully request that the Examiner reconsider and examine claims 45 and 55.

Rejections under 35 U.S.C. §103

The Examiner rejected claims 41, 44, 46, 55 and 56 under 35 U.S.C. §103, alleging that the claims are unpatentable over Yan, et al

(PNAS, 1997, 94:5296) in view of Hale, et al(Cytokine, 1995, 7:26) and Heaney, et al(Blood, 1993, 82:1945). The Examiner alleged that Yan et al teach that amyloid beta peptide binds to the receptor for RAGE. The Examiner alleged that they also demonstrated that binding of amyloid to the RAGE receptor induces oxidative stress, activation of microglia, and activation of inflammatory pathways involving transcription factor NF-κB. The Examiner alleged that they further suggest that these processes may contribute to the cellular pathologies seen in Alzheimer's disease. The Examiner stated that they do not teach inhibition of this binding event. The Examiner stated that Hale, et al. teach inhibition of tumor necrosis factor using the soluble form of its receptor. The Examiner stated that elevated levels of TNF are significant in the pathology of sepsis and the circulatory collapse that can result from sever sepsis. The Examiner stated that soluble receptors for TNF were shown to be effective in inhibiting the binding to TNFα to the cell-surface receptors. The Examiner stated that sTNFR's were also effective in inhibiting the effects of TNF in culture as well as in several models of sepsis in mice and baboons (p. 27 and 33). The Examiner stated that they further suggest that the endogenous "soluble receptors may be part of a negative feedback mechanism to inhibit the biological effects of TNF". The Examiner stated that Heaney and Golde give numerous examples in which soluble receptors are involved in intercellular signaling (p. 1946, for example). The Examiner stated that they discuss the consequences of such signaling to disease states, and suggest multiple roles for soluble receptors: for example, to temporarily inhibit or confer

sensitivity to a ligand (p. 1947). The Examiner stated that they conclude their discussion by stating that "construction and development of soluble receptors as pharmaceuticals may be useful to specifically inhibit or facilitate hormone action in disease states." The Examiner stated that it would have been obvious to a person of ordinary skill in the art at the time the invention was made to use the soluble form of RAGE to inhibit binding of an amyloid peptide that is known to bind the membrane-bound form of RAGE. The Examiner stated that the teachings of Yan et al., demonstrated binding of β-amyloid to the receptor for AGE on the surface of cells. The Examiner stated that binding triggers cascade of events that may be important in generating oxidative stress in cells expressing RAGE. The Examiner stated that an obvious way to inhibit such an event is to use the soluble form of the receptor to "out-compete" the endogenous binding. The Examiner stated that the literature gives examples of soluble receptors and examples wherein disease states have been successfully ameliorated using soluble receptors. The Examiner stated that the person of ordinary skill in the art would have been motivated to try to inhibit such binding events as those in the instant application. The Examiner stated that he would have reasonably expected success using a soluble form of the receptor to "tie-up" ligand, because he knows from the literature that the ligand binds the receptor. Furthermore, the Examiner stated that there are examples in the literature where similar methods were used to bind ligand and thus affect the outcome of a disease triggered by a binding event.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants contend that the cited references, namely Yan et al. in view of Hale et al. and Heaney et al. do not render obvious the claimed invention.

Initially, applicants point out that claim 41 recites as follows: "A method of inhibiting the binding of a β-**sheet fibril** to **RAGE** on the surface of a cell of a subject, wherein the cell is located outside the central nervous system of the subject, which comprises contacting the cell with a compound that inhibits binding of the β-sheet fibril to RAGE." [Emphasis added]

Yan et al. does not teach that a β-sheet fibril binds to RAGE on any cell and fail to suggest any method of inhibiting binding of β-sheet fibrils to RAGE. The Examiner concedes that Yan et al. "do **not** teach inhibition of this binding event," i.e. between amyloid-β and RAGE. Therefore there is no motivation in Yan et al. to use an inhibitor of RAGE such as sRAGE to inhibit such binding, let alone for the claimed purpose, i.e. inhibiting the binding of a β-**sheet fibril** to **RAGE**.

To compensate for the lack of any disclosure of inhibition of β-sheet fibril binding to RAGE in Yan et al., the Examiner relies on Hale et al. and Heaney et al. to support the role of a soluble form of a receptor inhibiting receptor mediated activation. Applicants contend that Hale et al. nor Heaney et al. do **not** offer a reasonable expectation of success regarding what is missing from

Yan et al., i.e. the inhibition of β-sheet fibril binding to RAGE, and do **not** render obvious the claimed invention.

Hale et al. do **not** render obvious the claimed invention. Hale et al. fail to teach a predictable inhibitory role for any soluble receptor, much less any predictable **inhibition** of **β-sheet fibril** binding to **RAGE** by a RAGE inhibitor such as **sRAGE**. The unpredictable inhibitory nature of soluble receptors is disclosed in Hale et al. as follows: "the functional dichotomies in the rsTNF-R family may be analogous to those described in the IGF-binding protein family(IGF-bp)," and "evidence acquired in various biological systems indicates that IGF-bp family members can function either as **inhibitors** or **enhancers** of IGF activity." [Emphasis added]. See page 33, second column. Hale et al. go on to recite that "the two soluble receptors of TNF-α (i.e. TNF-R's) may function analogously depending on the kinetics of their release, concentrations achieved in the various tissue compartments, and the physiological responses being studied." Therefore, while Hale et al. may at most teach of an activity of soluble type I and type II TNF receptors in a particular biological context, they clearly fail to teach a predictable inhibitory role for any soluble receptor, much less any predictable inhibition of β-sheet fibril binding to RAGE by a RAGE inhibitor such as sRAGE. Accordingly, applicants contend that because of the unpredictable nature of the inhibitory role of soluble receptors, Hale et al. fail to demonstrate a reasonable expectation of success regarding the inhibition of β-sheet fibril binding to RAGE and do **not** render obvious the claimed

invention.

Heaney et al. do **not** render obvious the claimed invention. Heaney et al. fail to teach a predictable inhibitory role for any soluble receptor/receptor pair, much less any predictable **inhibition** of β-**sheet fibril** binding to **RAGE** by a RAGE inhibitor such as **sRAGE**. In fact, Heaney et al. describe three possible models of soluble receptor function, two of which have no inhibitory capacity, and recite that "the physiologic role of the soluble (hormone) receptors are **incompletely understood** and may fall into three broad categories." [Emphasis added]. See page 1946, first column. Further discussing the subset of such broad categories of soluble hormone receptors which may have an inhibitory capacity, Heaney et al. recites that "it is uncertain whether soluble receptors, which are largely isoforms of low-affinity receptor subunits, effectively compete for ligand against multi-subunit receptors that have binding affinities that are often more than an order of magnitude higher." See page 1946, second column. Therefore, while Heaney et al. may at most teach that a subset of soluble hormone receptors may be inhibitory, they fail to teach a predictable inhibitory role for even these soluble receptors(i.e. a subset of soluble hormone receptors), much less any degree of predictability regarding the **inhibition** of β-**sheet fibril** binding to **RAGE** by a RAGE inhibitor such as sRAGE. Accordingly, applicants contend that because of the unpredictable nature of the inhibitory role of soluble receptors, Heaney et al. fail to demonstrate a reasonable expectation of success regarding the inhibition of β-sheet fibril binding to RAGE

and do **not** render obvious the claimed invention.

The applicants contend that the cited references, namely Yan et al. in view of Hale et al. and Heaney et al. do **not** demonstrate a reasonable expectation of success regarding the inhibition of β-sheet fibril binding to RAGE and do **not** render obvious the claimed invention. Accordingly, applicants contend that these comments obviate the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. § 112, first paragraph

The Examiner rejected claims 30-32 and 57-58 under 35 U.S.C. 112, first paragraph, because the subject matter was allegedly not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Examiner alleged that the specification is not enabling for the limitations of the claims wherein a method of inhibiting binding of a peptide that forms amyloid to a membrane-bound receptor for AGE is described. The Examiner alleged that claims 30-32, and 57-58 are drawn to a method of inhibiting binding of an amyloid peptide to a membrane-bound receptor for AGE. The Examiner stated that the specification discloses using the soluble form of the receptor to inhibit binding of amyloid to RAGE by concentration-dependent competition binding. The Examiner stated that experiments were described in which inhibition of binding between amyloid and RAGE was measured in PC12 cells transfected with RAGE. The Examiner

stated that the disclosure also described applying sRAGE to mouse splenic cells and subsequently measuring the changes in amyloid formation and changes in NfκB and interleukins. The Examiner stated that In re Wands, 8USPQ2d, 1400 (CAFC 1988) page 1404, the factors to be considered in determining whether a disclosure would require undue experimentation include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. The Examiner alleged that a sufficient amount of direction or guidance is lacking in claims 30-32, and 57-58. The Examiner stated that the specification gives examples wherein binding of amyloid by sRAGE in splenic cells or RAGE-transfected PC12 cells is inhibited in culture. However, the Examiner alleged that nowhere in the specification is a nexus described between inhibition of binding of amyloid and a disease state. The Examiner stated that the examples are directed to methods of inhibiting binding of any amyloid peptide to RAGE on cultured or isolated cells. The Examiner stated that later transduction events *are* described and measured: specifically the subsequent increase in NfκB and interleukins after binding of amyloid to cells. However, the Examiner alleged that the relationship between these early events and disease is poorly understood. In addition, the Examiner alleged that Alzheimer's disease therapy is highly unpredictable, and using the methods described to obtain *any* clinical effect would

require a large amount of experimentation. In summary, the Examiner alleged that the specification does not provide a description of a repeatable process of inhibiting binding of an amyloid peptide to RAGE on cultured or isolated cells in such a way as to modulate a disease state involving "β-sheet fibrils". In addition, the Examiner alleged that the predictability of the art is very low with regard to the results of inhibiting binding of an amyloid peptide to RAGE in a mammal with a disease involving a "β-sheet fibril" in the manner specified. The Examiner alleged that for this reason undue experimentation would be required to determine effective methods of inhibiting binding of an amyloid peptide to RAGE to ameliorate a disease state.

In response, applicants respectfully traverse Examiner's above rejection. Applicants contend that the *in vitro* and *in vivo* data taken as a whole demonstrate the ability of the disclosed invention to modulate a disease state involving β-sheet fibrils and therefore the invention is enabled.

In vitro data

The *in vitro* data demonstrate that RAGE interacts specifically with Aβ fibrils and that the blockade of RAGE completely suppressed symptoms of cellular stress such as fibril-dependent NF-κB activation and DNA fragmentation. The specification recites that in a purified *in vitro* system, RAGE interacts specifically with Aβ fibrils while a peptide containing the reverse sequence of Aβ did not bind RAGE, nor did several other control peptides of

hydrophobicity similar to Aβ. See page 63, lines 18-35 and page 64, lines 1-6. In order to relate RAGE engagement by amyloid fibrils to events occurring at the cell surface and their consequences for cellular behavior, the specification recites that stably-transfected PC12 cell-RAGE transfectants (PC12/RAGE) that overexpress wild-type receptor displayed increased total RAGE antigen by immunoblotting and elevated levels of cell surface RAGE by immunohistochemistry and increased MAP kinase pathway and NF-κB activation by ERK ½ immunoblotting. See page 67, lines 14-35 and page 68, lines 1-22. Further the specification recites that blockade of RAGE suppressed fibril-dependent NF-κB activation and DNA fragmentation completely. See page 72, lines 22-25. Therefore, the *in vitro* data demonstrate that RAGE interacts specifically with Aβ fibrils and that the blockade of RAGE completely suppressed symptoms of cellular stress such as fibril-dependent NF-κB activation and DNA fragmentation.

In vivo data

In a murine model of systemic amyloidosis, blockade of fibril-RAGE interactions with sRAGE *in vivo* suppressed symptoms of Alzheimer's disease and systemic amyloidosis such as cellular stress and amyloid A fibril accumulation. The specification recites as follows: "In a model of systemic amyloidosis, blockade of fibril-RAGE interaction *in vivo* suppressed cellular stress and amyloid A fibril accumulation." See page 57, lines 17-20. Further, the specification recites that in an *in vivo* model of systemic amyloidosis, blockade of fibril-RAGE interaction suppressed

cellular stress and amyloid A fibril accumulation, suggesting that cell surface RAGE is a focal point for interaction with fibrils, which renders amyloid pathogenic by a receptor-dependent mechanism. See page 74, lines 1-31. In addition, the specification teaches that there are common denominators of fibrilar pathologies, such as Alzheimer's disease and systemic amyloidosis, principally from accumulated debris in the form of fibrils encroaching on normal structures and that the identification of RAGE as a signal transduction receptor for β-sheet fibrils demonstrates a means through which fibril formation affects cellular interactions. See page 80, lines 18-32. Therefore the specification teaches that in a murine model of systemic amyloidosis, blockade of fibril-RAGE interactions with sRAGE *in vivo* suppressed symptoms of Alzheimer's disease and systemic amyloidosis such as cellular stress and amyloid A fibril accumulation. Accordingly, applicants contend that the *in vitro* and *in vivo* data taken as a whole demonstrate the ability of the disclosed invention to modulate a disease state involving β-sheet fibrils and therefore the invention is enabled. Applicants contend that these comments obviate the above objection and respectfully request that the Examiner reconsider and withdraw this objection.

Rejection under - 35 USC §112, first paragraph

The Examiner rejected claims 41, 44, 46 and 55-56 under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for inhibiting binding of β-amyloid to RAGE *in vitro*, allegedly does not reasonably provide enablement for inhibiting

binding of amyloid to RAGE *in vivo*. The Examiner alleges that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims. The Examiner stated that the claims are directed to the use of soluble RAGE to inhibit binding of amyloid peptide to RAGE. The Examiner stated that the specification discloses methods for using the soluble receptor for AGE as an inhibitor of binding of a β-sheet fibril to the membrane-bound RAGE. The Examiner alleged that the scope of the patent protection sought by the Applicant is defined by the claims fails to correlate reasonably with the scope of enabling disclosure set forth in the specification for the following reasons: The Examiner stated that the specification discloses an enabled utility for soluble RAGE as to be used to inhibit binding of amylin, β-amyloid or related peptides to the membrane-bound receptor for AGE in PC12 cells. The Examiner stated that the specification reads on a curative or preventative therapy for certain dementias as Alzheimer's disease. However, the Examiner stated that Alzheimer's disease is a highly complex disorder that may take *years* to develop (Perlman, et al. Neurobiology of Disease, pp 307-318, esp. Pp 310-311). In addition, the Examiner stated that multiple neuronal cell types are involved (pp 315-317), and several protein types contribute to plaques and tangles (pp 316-317). The Examiner stated that despite several lines of research ranging from genetics and immunology to pharmacology and cognitive sciences, and despite the fact that the clinical diagnosis is relatively unambiguous, at least in later

stages, Alzheimer's disease is still seen as a largely incurable and untreatable disease (pp. 310-313). Furthermore, the Examiner alleged that there is no discussion in the instant application of how to administer sRAGE in humans and how to measure the clinical effects. The Examiner alleged that there are no discussions of routes of administration, side effects, or dosages needed. The Examiner stated that In re Wands, 8USPQ2d, 1400 (CAFC 1988) page 1404, the factors to be considered in determining whether a disclosure would require undue experimentation include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. The Examiner alleged that due to the large quantity of experimentation required to determine how to use soluble RAGE to inhibit amyloidosis *in vivo*, the lack of direction or guidance in the specification regarding same (e.g., the lack of guidance regarding specific activity of sRAGE in humans), the lack of working examples to same, the state of the art showing the unpredictability of treating dementias, and the breadth of the claims which embrace *in vivo* methods, undue experimentation would be required of the skilled artisan and use the claimed invention in its full scope.

In response, applicants respectfully traverse Examiner's rejection of the claims. Applicants contend that the invention is enabled

and that the specification teaches those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation.

In vivo use of sRAGE

The specification is enabled for the *in vivo* use of sRAGE to inhibit amyloidosis. The specification recites that in a murine model of systemic amyloidosis C57BL6/J mice treated with recombinant murine sRAGE demonstrate a dose-dependent supression of splenic amyloid burden (up to 60%) compared to control mice. See page 76, lines 31-35. In addition, the specification recites that "administration of fragments [$F(ab')_2$] prepared from blocking polyclonal antibody to RAGE to mice undergoing treatment with amyloid enhancing factor/silver nitrate resulted in suppression of markers of cellular stress and amyloid accumulation in the spleen similarly to what was observed in animals treated with sRAGE." See page 77, lines 15-20. Therefore, the specification recites that in a murine model of systemic amyloidosis, administration of sRAGE decreases splenic amyloid burden in a dose-dependent manner. Accordingly, the specification is enabled for the *in vivo* use of sRAGE to inhibit amyloidosis.

State of the art of transgene behavior

The specification is enabled for the treatment of dementias such as those associated with Alzheimer's disease (hereinafter AD) without undue experimentation. Various papers support the idea that transgenic mice with Alzheimer's disease-type pathology

(hereinafter AD-type pathology) may be useful for identifying methods of treatment of dementias associated with Alzheimer's disease in a human subject.

In support, applicants attach hereto as **Exhibit A** a copy of a paper by Hsaio et al.(Science 274:99-102, 1996), entitled "Correlative Memory Deficits, Aβ Elevation, and Amyloid Plaques in Transgenic Mice" states that they have produced a strain of transgenic mice which overexpress the 695-amino acid isoform of human Alzheimer β-amyloid(Aβ) precursor protein and that the "correlative appearance of behavioral, biochemical, and pathological abnormalities reminiscent of Alzheimer's disease in these transgenic mice suggests new opportunities for exploring the pathophysiology and neurobiology of this disease." See page 99, abstract. Specifically, the paper shows that transgenic mice expressing 5-fold and 14-fold increase in expression of the 695-amino acid isoform of human APP containing a lys670Asn, Met671Leu mutation had normal learning and memory in spatial reference and alteration tasks at three months of age but developed plaques and showed impairment by 9-10 months of age. See page 99, figure 1 and page 100, figure 2. The paper further states that their results "demonstrate the feasibility of creating transgenic mice with robust behavioral and pathological features resembling those found in AD." See page 102, column 1. Accordingly, the applicants contend that this paper shows that transgenic mice which overexpress human APP develop Aβ plaques and behavioral deficits similar to those observed in human AD subjects and therefore model

AD in a human subject.

In further support, applicants also attach hereto as **Exhibit B** a copy of a paper by John Hardy(Proc. Natl. Acad. Sci. 90:2095-2097, 1997), entitled "The Alzheimer family of diseases: Many etiologies, one pathogenesis?" which states that mutations in the APP, PS1 and PS2 genes, singly or in combination, alter APP processing such that an increased amount of Aβ42 is produced causing AD-type pathology in cell culture, transgenic mice, and human subjects with PS-encoded Alzheimer's disease. See page 2095, first column. The paper further states that pathological analysis show that Aβ42 is deposited early and selectively in plaques that are characteristic of AD and that these *in vitro* cell culture and *in vivo* mouse data "give a single, coherent picture of the pathogenesis of the disease" and "provide the strongest possible evidence that the amyloid cascade hypothesis for the etiology and pathogenesis of AD is correct." See page 2095, first column. The authors conclude by stating that with regards to the development of AD in humans, "we now know several causes of the disease(all genetic so far), and all of these causes have pointed toward the path of APP metabolism and deposition as being the early event in the disease pathogenesis." See page 2096, first column. Accordingly, applicants contend that this paper shows the following: 1)that an increased amount of Aβ42 in transgenic mice create plaques that are characteristic of AD; 2)an increased amount of Aβ42 is observed in human subjects with PS-encoded AD; and 3)that the causes of AD have pointed toward the path of APP metabolism and deposition causing plaque formation as

β attenuates Alzheimer-disease-like pathology in the PDAPP mouse" which demonstrate the effect of Aβ immunization on plaque load in transgenic mice with Alzheimer's disease-type neuropathologies and state "that such immunization reduced β-amyloid plaque formation and may be effective in treating Alzheimer's disease." See page 247, abstract. The paper also states that "the Aβ42-immunized mice never developed the neurodegenerative lesion that typify the progression of AD-type pathology in this model." See page 175, column 2. The paper further states that these data are the first report of "a clinically relevant treatment that reduces the progression of AD-like neuropathology in a transgenic animal model of the disease," and "that it is not unreasonable to expect that a similar reduction of neuropathology in AD patients would be of clinical benefit." See page 177, column 1. Accordingly, applicants contend that this paper shows that the reduction of β-amyloid plaque treats AD-type pathology in a transgenic mouse model of AD and may therefore be effective in treating a human subject with AD.

In support, applicants further attach hereto as **Exhibit E** a copy of a paper by Weggen et al.(Nature 414:212-216, 2001), entitled "A subset of NSAIDS lower amyloidogenic Aβ42 independently of cyclooxygenase activity" which states that "epidemiological studies have documented a reduced prevalence of Alzheimer's disease among user of nonsteroidal anti-inflammatory drugs(NSAIDS)." The paper further states that two selective COX inhibitors, i.e. ibuprofen and indomethacin, have "reduced amyloid plaque pathology in a mouse model(Tg2576 transgenic mice) of Alzheimer's disease" and "seemed

being the early events in AD pathogenesis. Therefore, the applicants maintain that transgenic mice, such as the TgAPPsw+/- mice used in the present application, which exhibit an increase in Aβ42 and plaque formation provide a useful model for the treatment of AD in a human subject.

Applicants further attach as **Exhibit C** a copy of a paper by Marina R. Picciotto and Kevin Wickman(Physiol. Rev. 78: 1131-1163, 1998), entitled "Using Knockout and Transgenic Mice to study Neurophysiology and Behavior" which states that "mice have been generated that develop plaques and show behavioral consequences linked to mutant APP expression, establishing a connection between abnormal APP expression and cognitive impairment." See page 1154, column 2. The paper further states that transgenic mice expressing human APP and fAD-linked PS1 mutants under the control of the hamster prion promoter exhibited significant Aβ42 overproduction in the brain and that these "mouse models have begun to identify the mechanisms underlying the pathology associated with Alzheimer's disease." See page 1155, column 1. Accordingly, the Picciotto and Wickman paper indicates that transgenic mice which abnormally express mutant APP develop plaques and behavioral hallmarks associated with AD in a human subject and therefore have begun to identify the mechanisms underlying development of AD in a human subject.

Applicants further attach as **Exhibit D** a paper by Schenck et al.(Nature 400:173-177, 1999), entitled "Immunization with amyloid-

to slow the cognitive decline in [human] patients with Alzheimer's disease." See page 213, column 1 and page 214, column 1. Accordingly, applicants contend that this paper shows that a subset of NSAIDS treated AD-type pathology in transgenic mice and symptoms of Alzheimer's disease in human patients.

Administration of sRAGE to humans

The applicants maintain that the administration of sRAGE to humans and measurement of the clinical effects of such administration would be obvious to one skilled in the art. The specification recites that in a murine model of systemic amyloidosis C57BL6/J mice were treated with recombinant murine sRAGE, saline or mouse serum albumin injected intraperitoneally once daily starting at day -1 (day 0 indicates the start of AEF/SN) and continuing up to day 4. See page 62, lines 26-30. Further, the specification recites as follows: "dose-dependent suppression of splenic amyloid burden (up to 60%) was observed in sRAGE-treated AEF/SN mice, compared with animals receiving vehicle (mouse serum albumin) alone. See page 76, lines 31-35. In addition, the specification recites that splenic IL-6 antigen was strongly elevated in AEF/SN-treated mice and taken together with the accumulation of splenic amyloid in such AEF/SN-treated mice, these data show a strong association between increased tissue amyloid burden and cellular stress. See page 74, lines 23-31. Therefore, the specification teaches that sRAGE measurably treats systemic amyloidosis in a dose dependent manner. Accordingly, applicants maintain that the administration of sRAGE to humans and measurement of the clinical effects of such

administration would be obvious to one skilled in the art. Accordingly, applicants maintain that the specification teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation. Applicants contend that these comments obviate the above rejection and respectfully request that the Examiner reconsider and withdraw this objection.

Rejection under - 35 U.S.C. §112, second paragraph

The Examiner rejected claims 30, 31, 32, 41, 44, 46, 55, 56, 57, 58 as being indefinite for allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Examiner alleged that the claims encompass a method of using sRAGE for preventing the interaction of an amyloid-forming peptide with RAGE. However, the Examiner alleged that one skilled in the art cannot determine the metes and bounds of the claimed invention because there is no recognized structural or functional determinants in the claims such that the molecules encompassed can be distinguished from any other molecule. The Examiner alleged that aside from the art-recognized names of the molecules used for the claimed invention, there is nothing to distinguish them from similar peptides from other species, nor from mutants and variants.

In response, applicants respectfully traverse Examiner's rejection of the claims as being indefinite. The specification identifies the metes and bounds of the claimed invention by disclosing the

structural and functional determinants of sRAGE such that it can be distinguished from other molecules.

The specification is not indefinite. The specification recites that a compound used to inhibit the binding of a β-sheet fibril to RAGE on the surface of a cell may be sRAGE or a fragment thereof including the isolated peptide having an amino acid sequence corresponding to the V-domain of RAGE. See page 18, lines 31-35 and page 19, lines 25-28. Further, the specification recite that Neeper et al.(1992) disclose the full length amino acid sequence of RAGE as well as the highly conserved 120 amino acid V-domain. See page page 21, lines 1-11. Therefore, the specification discloses the highly conserved structure and function of RAGE, including sRAGE, the V-domain of sRAGE or fragments thereof so disclosed in the invention. Accordingly, the specification is not indefinite. Applicants contend that these comments obviate the above rejection and respectfully request that the Examiner reconsider and withdraw this objection.

Summary

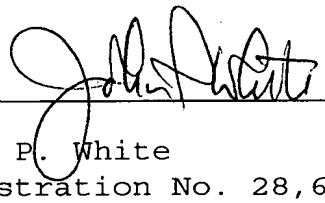
For the reasons set forth hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection and rejection and earnestly solicit allowance of the now pending claims, i.e. claims 41-58.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned

attorney invite the Examiner to telephone him at the number provided below.

No fee, other than the enclosed $460.00 fee for a three-month extension of time, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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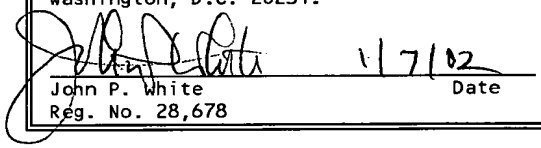
I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.



1/7/02
Date

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P# 4

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Form PTO-1449 U.S. Department of Commerce Patent and Trademark Office	Atty. Docket No. 59472/JPW/SHS	Serial No. 09/374,213
INFORMATION DISCLOSURE CITATION (Use several sheets if necessary)	Applicants David Stern et al.	
	Filing Date August 13, 1999	Group 1647

U.S. PATENT DOCUMENTS

Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date if Appropriate

FOREIGN PATENT DOCUMENTS

		Document Number	Date	Country	Class	Subclass	Translation	
							Yes	No

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

SLW		Akama, T. Keith, et al., (1998) "Amyloid β-peptide stimulates nitric oxide production in astrocytes through an NFκB-dependent mechanism," Proc. Natl. Acad. Sci. 95:5795-5800 ~~(Exhibit 1);~~
SLW		Behl, C., et al., (1994) "Hydrogen Peroxide Mediates Amyloid β Protein Toxicity," Cell 77:817-827 ~~(Exhibit 2)~~;
SLW		Combs, K. Colin, et al. (1999) "Identification of Microglial Signal Transduction Pathways Mediating a Neurotoxic Response to Amyloidogenic Fragments of β-Amyloid and Prion Proteins," Journal of Neuroscience 19(3)928-939 ~~(Exhibit 3);~~
SLW		Forloni, Gianluigi, et al. (1996) "Amyloid in Alzheimer's Disease and Prior-Related Encephalopathies: Studies With Synthetic Peptides," Progress in Neurobiology 49:287-315 ~~(Exhibit 4);~~
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SLW		Inagaki, Fuyuhiko, et al. (1978) "Conformation of Erabutoxins a and b in Aqueous Solution as Studied by Nuclear Magnetic Resonance and Circular Dichroism," 89:433-443 ~~(Exhibit 6);~~
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EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if citation considered, whether or not citation is in conf citation if not in conformance and not considered. Include copy of this form

Sandra Weigel 5/26/01

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23. K.S., U.P., D.L., and C.R.B. are recipients of grants of the Sonderforschungsbereich 322 from the Deutsche Forschungsgemeinschaft. G.H.G. is supported by a PHS grant awarded to the Stanford University Program in Cancer Biology. M.R.L. is a Leukemia Society of America Scholar, and research in his laboratory is supported by grants from the NIH and a grant from the Council for Tobacco Research. S.D. is supported by a grant from the National Cancer Institute and by the Howard Hughes Medical Institute.

3 June 1996; accepted 20 August 1996

Correlative Memory Deficits, Aβ Elevation, and Amyloid Plaques in Transgenic Mice

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Transgenic mice overexpressing the 695-amino acid isoform of human Alzheimer β-amyloid (Aβ) precursor protein containing a $Lys^{670} \rightarrow Asn$, $Met^{671} \rightarrow Leu$ mutation had normal learning and memory in spatial reference and alternation tasks at 3 months of age but showed impairment by 9 to 10 months of age. A fivefold increase in Aβ(1–40) and a 14-fold increase in Aβ(1–42/43) accompanied the appearance of these behavioral deficits. Numerous Aβ plaques that stained with Congo red dye were present in cortical and limbic structures of mice with elevated amounts of Aβ. The correlative appearance of behavioral, biochemical, and pathological abnormalities reminiscent of Alzheimer's disease in these transgenic mice suggests new opportunities for exploring the pathophysiology and neurobiology of this disease.

Alzheimer's disease (AD), the most common cause of dementia in aged humans, is a disease of unknown etiology. Amyloid plaques are routinely used for diagnosing AD in brain tissue (*1*), even though other histologic changes such as neurofibrillary tangles, synaptic and neuronal loss, and dystrophic neurites are also usually present and sometimes correlate better with dementia (*2*, *3*). The amyloid in senile plaques is composed of Aβ, a 39– to 43–amino acid protein derived from the larger amyloid precursor protein (APP). Small numbers of classic senile plaques develop in the brain with age, but large numbers of senile plaques are found almost exclusively in patients with Alzheimer's type dementia. A diagnosis of AD is made only if both cognitive deterioration and senile plaques are present (*4*). APP isoforms resulting from alternative splicing form a set of polypeptides ranging from 563 to 770 residues in length. The most abundant of these, APP_{695}, is predominantly expressed in neurons (*5*) and lacks a Kunitz-protease inhibitor (KPI) domain present in the APP_{751} and APP_{770} isoforms. Five mutations in APP, all located in or near the Aβ domain, have been identified in families with early-onset AD (*6–10*).

Transgenic mice (Swiss Webster × C57B6/DBA2) expressing three isoforms of mutant APP ($Val^{717} \rightarrow Phe$) with an overrepresentation of KPI-containing isoforms showed Alzheimer-type neuropathology, including abundant thioflavin S–positive Aβ deposits, neuritic plaques, synaptic loss, astrocytosis, and microgliosis (*11*), but deficits in memory and learning have not yet been reported. Transgenic mice (JU) expressing human wild-type APP_{751} showed deficits in spatial reference and alternation tasks by 12 months of age (*12*). However, only 4% of aged (≥12 months) transgenic mice exhibited Aβ deposits, and these were rare and diffuse and did not stain with Congo red dye (*13*). Transgenic mice (FVB/N) overexpressing wild-type and variant human or mouse APP_{695} developed a central nervous system disorder that involved most of the corticolimbic regions of the brain (except the somatosensorimotor area) and resembled an accelerated naturally occurring senescent disorder of FVB/N mice (*14*). Parameters that influence the phenotype of transgenic mice expressing APP include host strain, APP primary structure, and extent of APP expression (*14*). We investigated the effects of APP overexpression in C57B6/SJL F_2 mice backcrossed to C57B6 breeders because of their greater longevity compared with FVB/N mice expressing identical transgenes.

Human APP_{695} containing the double mutation $Lys^{670} \rightarrow Asn$, $Met^{671} \rightarrow Leu$ (K670N,M671L; APP_{770} numbering), which was found in a large Swedish family with early-onset AD (*10*), was inserted into a hamster prion protein (PrP) cosmid vector (*15*) in which the PrP open reading frame (ORF) was replaced with the variant APP ORF [see (*14*)]. The resulting mice, Tg(HuAPP695.K670N-M671L)2576, produced 5.56 ± 0.33 units (mean ± SEM; 73-day-old mice) to 5.76 ± 0.74 units (430-day-old mice) of transgenic brain APP expression, where a unit of expression is equivalent to the amount of endogenous mouse APP in nontransgenic (control) littermates (Fig. 1). Transgenic APP expres-

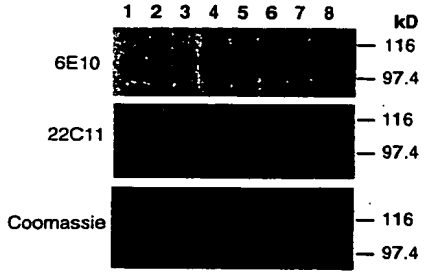

Fig. 1. Brain APP immunoblot of young and old Tg^+ mice and nontransgenic control mice with 6E10 (*24*), which recognizes human but not mouse APP, and 22C11 (Boehringer Mannheim), which recognizes both human and mouse APP. Lanes 1 to 3, nontransgenic mice; lanes 4 to 6, 73-day-old mice; lanes 7 and 8, 430-day-old mice. Detailed methods for APP quantitation were described previously (*14*); antibody binding was revealed with ^{35}S-labeled protein A instead of ^{125}I-labeled protein A.

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sion appeared to remain unchanged between 2 and 14 months of age.

Two groups of 7 to 9 transgene-positive (Tg^+) mice and 10 to 11 transgene-negative (Tg^-) control littermates underwent spatial alternation testing in a Y-maze at 3 and 10 months of age. Three groups of 9 to 13 Tg^+ mice and 10 to 14 Tg^- littermates underwent spatial reference learning and memory testing in the Morris water maze (*16*) at 2, 6, and 9 to 10 months of age. The test experience for each set of mice was novel, and all mice were tested in a coded manner. The 9- to 10-month-old mice were N_1-generation mice (C57B6 × C57B6/SJL F_2); the 2- and 6-month-old mice were N_2-generation mice (C57B6 × C57B6 × C57B6/SJL F_2). A subset of the N_2-generation mice (8 transgenic and 10 control mice) were retested at 12 to 15 months of age.

When transgenic and control mice were given a choice of entering either of two arms in a Y-maze, they tended to alternate their choices spontaneously. Ten-month-old transgenic mice, however, showed significantly less tendency ($P < 0.03$) than did age-matched control mice to alternate the arms on successive choices (Fig. 2F). The behavior of the older transgenic mice on the spatial alternation task was characteristic of animals with damage to the hippocampal formation (*17*).

Nine- to 10-month-old transgenic mice were also impaired in their performance in the water maze relative to age-matched controls (*18*) (Fig. 2). The performance of transgenic mice trained and tested at 2 or 6 months of age was not significantly different from that of age-matched control mice on most measures. The amount of time taken by the mice to reach the hidden platform (the escape latency) did not differ between 2-month-old transgenic and control mice at any point during training, whereas the latency was significantly different on every day for 9- to 10-month-old mice (*19*). Six-month-old transgenic mice differed from controls in escape latency only on the last day of training. After the last training day (day 6), all mice were given a probe trial, in which they swam in the pool for 60 s with the platform removed (*20*). One measure of the animals' knowledge of platform location is the percentage of the 60-s swim spent in the target quadrant (the quadrant that held the platform during training; Fig. 2B). Because the platform is placed in the center of the target quadrant during training, an additional measure that has proven especially useful for mice involves recording the number of times they cross the center of each quadrant. The number of times each mouse crossed the center of the target quadrant (platform crossings; Fig. 2C) and the percentage of total quadrant center crossings that were in the target quadrant were both significantly different [21.5 ± 5.2% for transgenic mice versus 36.1 ± 3.9% for control mice ($P < 0.05$), where 25% is performance at the level of chance] (Fig. 2D) for 9- to 10-month-old transgenic mice compared with age-matched controls.

When 12- to 15-month-old N_2-generation transgenic mice were retested in the water maze (after rearranging the extramaze cues), they showed significantly impaired performance ($P < 0.05$) compared with control littermates on escape latencies after the fifth trial block and on probe trials given after the sixth and ninth trial blocks. These data suggest that the age-related learning impairment seen in N_1-generation Tg^+ mice can occur despite further genetic dilution of the SJL strain. Although the escape latencies of the transgenic N_2-generation mice were significantly longer than those of their control littermates, they were also shorter than those of naïve Tg^+ mice of comparable age. Thus, deficits in escape latency in aged transgenic mice are unlikely to result from difficulty in swimming, as aged mice given sufficient practice can swim as well as younger mice.

Because it is possible that the performance of older transgenic mice was attributable to sensory or motor impairments, we also tested 9- to 10-month-old mice on the visible-platform version of the water maze (Fig. 2E). Although differences in escape latency were evident on the second and fourth of four training days, there were no differences on day 1. These data suggest that although older transgenic mice may show generalized cognitive impairment, they are capable of performing as well as controls when both are relatively naïve. We

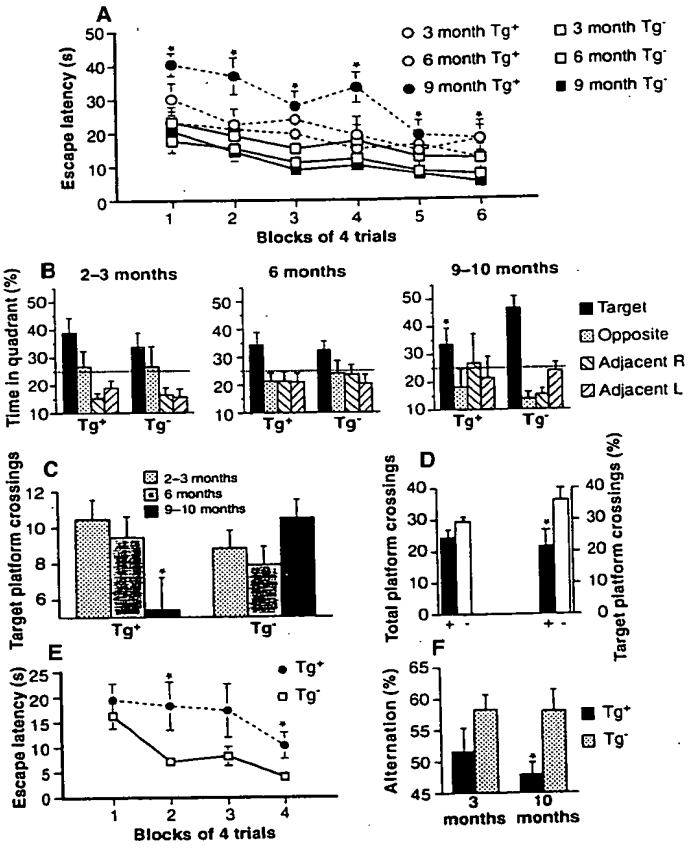

Fig. 2. Learning and memory tests of transgenic and control mice. Asterisks indicate measures in which transgenic mice differed significantly from controls ($P < 0.05$). **(A)** The latency to escape to the hidden platform in the water maze is impaired in Tg^+ mice relative to age-matched nontransgenic controls (*19*). Although the impairment increases with age, Tg^+ mice showed a consistent trend toward longer escape latencies than those of Tg^- controls. **(B)** After 24 trials (over 6 days) with the platform in its fixed location, mice were given a probe trial in which they swam for 60 s with the platform removed. Two- and 6-month-old Tg^- and Tg^+ mice spent significantly more than 25% of their time in the target quadrant, indicating that they had learned its location. Although 9- to 10-month-old control mice still searched selectively for the platform, older transgenic mice spent no more time in the target quadrant than in the other three quadrants, suggesting that they had not learned the platform's location (*20*). **(C)** The implications of (B) are supported by the observation that on probe trials, 9- to 10-month-old Tg^+ mice crossed what had been the exact location of the platform significantly less frequently than did age-matched Tg^- mice. **(D)** The bars on the left indicate that transgenic (+) mice did not differ from control (–) mice in the total number of platform locations crossed (that is, the centers of all four quadrants); the bars on the right show the significant difference between 9- to 10-month-old transgenic mice and controls on the percentage of total platform crossings that were over the target. **(E)** Nine- to 10-month-old Tg^+ mice were also impaired in swimming to a visible platform, although escape latencies did not differ significantly on the first visible-platform training trial. **(F)** Aged Tg^+ mice were impaired in their tendency to spontaneously alternate arm-entry in a Y-maze, another behavioral task sensitive to hippocampal damage.

also compared motor performance of the transgenic and control 9-month-old mice by scoring the total number of times during the probe trial that each mouse crossed imaginary platforms located in each of the four quadrants. If impaired mice swim normally but in a random pattern during probe trials, they should cross the center of all four quadrants combined as many times as would unimpaired mice; they will simply cross the target platform fewer times. If, on the other hand, they are impaired on probe trials simply because they are not swimming, there will be fewer total platform crossings. In fact, the total numbers of platform crossings for transgenic mice (24.4 ± 8.7, mean ± SEM) and control mice (29.5 ± 1.4) were not significantly different, which indicated that motor impairment was not a cause of poor performance in the water maze (Fig. 2D).

After behavioral testing, a subset of each group of mice was killed painlessly. One hemibrain was frozen for cerebral cortical Aβ measurements, and the other hemibrain was immersion-fixed for histopathological analysis. All brains were analyzed in a coded fashion. Measurements of Aβ(1–40) and of Aβ(1–42/43) were done with the use of either the Ban-50/Ba-27 or Ban-50/Bc-05 enzyme-linked immunosorbent assay (ELISA) systems (*21*, *22*). These measurements showed a fivefold increase in the concentration of Aβ(1–40) ($P = 0.03$, rank sum test) and a 14-fold increase in that of Aβ(1–42/43) ($P = 0.03$, rank sum test) between the youngest (2 to 8 months) and oldest (11 to 13 months) Tg^+ mice (Table 1). Thus, there was an association between significantly elevated amounts of Aβ and the appearance of memory and learning deficits in the oldest group of transgenic mice.

Classic senile plaques (with dense amyloid cores) and diffuse deposits were both present in all three mice with elevated Aβ, as determined by ELISA. The Aβ deposits were immunoreactive with antibodies recognizing Aβ(1–5) (*23*), Aβ(1–17) (*24*), Aβ(17–24) (*25*), Aβ(34–40) (*26*), Aβ(42/43) (*27*), and free Aβ42 (*28*). The same plaques were readily identified with multiple antibodies on adjacent sections and were not seen with preimmune or nonspecific ascites, and the immunoreactivity was eliminated by preabsorption with the relevant peptides (Fig. 3). Deposits could not be found in the older or younger controls or in the younger transgenic mice examined. The deposits were found in frontal, temporal, and entorhinal cortex, hippocampus, presubiculum, subiculum, and cerebellum, in a pattern similar to that reported by Games *et al.* (*11*). Dense amyloid plaques were most frequent in cortex, subiculum, and presubiculum. The dense amyloid deposits were readily detected with thioflavin S fluorescence and typically could also be labeled with Congo red to give the characteristic apple-green birefringence of classical amyloid (*29*). Some small deposits had the "Maltese cross" signature pattern of the amyloid cores found in AD brains. Under high magnification, the thioflavin S– and Congo red–positive amyloid plaques usually exhibited wisps or fibers radiating from the central mass, which was often ringed by glial nuclei with both astrocytic and microglial morphology. Glial fibrillary acidic protein–immunoreactive astrocytes were associated with amyloid deposition. Staining by the Gallyas silver method revealed dystrophic neurites surrounding dense core plaques.

In contrast to plaques from patients with sporadic AD, antibodies to β1 and to both free Aβ(42) and Aβ(34–40) (which preferentially recognizes x-40) labeled the ma-

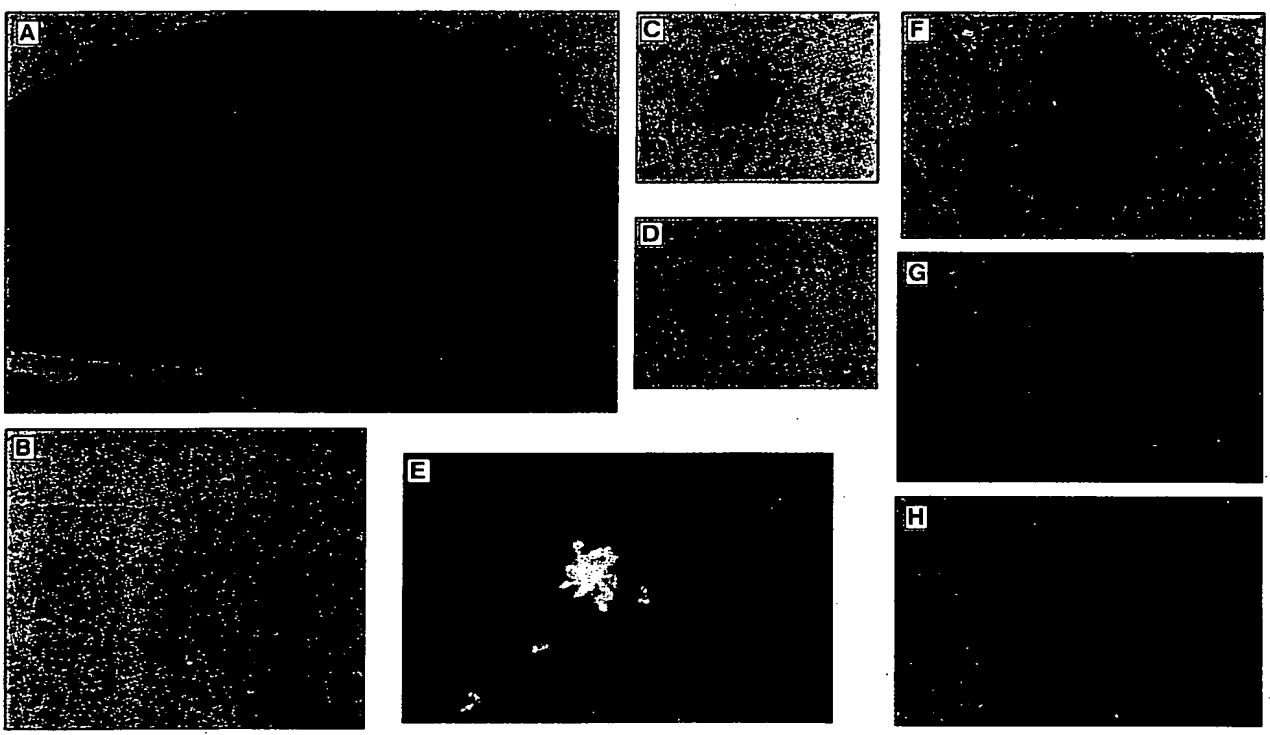

Fig. 3. Extracellular amyloid deposits in transgenic mice A01493 (age, 368 days) and A01488 (354 days) overexpressing human APP_{695} with the K670N,M671L mutation. (**A**) A01493, multiple plaques in the cerebral cortex and subiculum staining with 4G8 mAb. (**B**) A01493, inset from (A). (**C**) A01488, plaque in subiculum staining with 4G8 mAb. (**D**) A01488, plaque in section adjacent to (C) fails to stain with 4G8 mAb preabsorbed with Aβ(14–24). (**E**) A01488, plaques staining with thioflavin S. (**F**) A01488, plaque staining with Aβ(1) affinity-purified antiserum specifically recognizing the NH_2-terminus of Aβ. (**G**) A01488, plaque staining with Aβ(42) affinity-purified antiserum specifically recognizing the COOH-terminus of Aβ(1–42). (**H**) A01488, plaque staining with α40 affinity-purified antiserum specifically recognizing the COOH-terminus of Aβ(1–40). Magnifications: ×100 (A), ×250 (B), ×1000 (C, D, F, and G), ×640 (E), and ×500 (H).

Table 1. Concentrations of Aβ in transgenic and control mouse brains. Brain tissue was stained with monoclonal antibody (mAb) 4G8 (*25*), which recognizes both mouse and human Aβ. All amyloid deposits stained with 6E10 (*24*), which specifically recognizes human Aβ. No extracellular 6E10 staining was detected in three 105- to 106-day-old Tg^+ mice or one 155-day-old Tg^+ mouse (A01480, A01547, A01548, and Tg2576 founder). ++, 2 to 5 plaques per section; +++, 6 to 10 plaques per section; ++++, >10 plaques per section; –, no staining. Because all the pathological specimens were analyzed in a coded fashion, some nonspecific, equivocal staining that could not be blocked by preabsorption of the antibody with specific peptides was observed in some sections (indicated by ±).

Mouse number	Transgene	Age when killed (days)	Aβ(1–40) (pmol/g)	Aβ(1–42/43) (pmol/g)	Amyloid plaques
		Mice killed at 11 to 13 months of age			
A01484	+	361	325	219	+++
A01488	+	354	192	129	++
A01489	–	354	<2	<2	±
A01492	–	371	<2	<2	–
A01493	+	368	273	177	++++
A01495	–	354	<2	<2	–
A01496	–	354	<2	<2	±
Mean (±SEM) Aβ concentration in Tg^+ mice:			264 ± 38	175 ± 26	
		Mice killed at 6 to 8 months of age			
A01984	–	233	<2	<2	±
A01987	–	219	<2	<2	–
A01989	+	219	45	18	–
A02561	–	214	<2	<2	
A02595	–	207	<2	<2	
		Mice killed at 2 to 5 months of age			
A02428	–	139	<2	<2	–
A02429	–	139	<2	<2	
A02430	–	139	<2	<2	–
A02565	+	118	71	21	
A02900	–	85	<2	<2	
A03103	+	67	32	2	
A03107	+	67	45	10	
Mean (±SEM) Aβ concentration in Tg^+ mice:			48 ± 8	13 ± 4	

jority of deposits. This may reflect the $APP_{670-671}$ mutations, which greatly increase cleavage at the β1 site, leading to large concentrations of all fragments beginning with the β1 epitope. In contrast, the $Val^{717} \rightarrow$ Phe mutations increase the percentage of x-42 (*21*, *30*).

Our results demonstrate the feasibility of creating transgenic mice with robust behavioral and pathological features resembling those found in AD. Impairment in learning and memory became apparent in mice 9 months of age and older; this impairment was correlated with markedly increased amounts of Aβ and was accompanied by numerous amyloid plaques and Aβ deposits. We have demonstrated that an APP transgene lacking the KPI domain is also capable of engendering amyloid plaques in mice. The increase in the concentration of Aβ cannot be explained by a rise in transgenic APP expression, which appeared to remain unchanged with age. Concentrations of Aβ(1–42/43) rose more markedly than did those of Aβ(1–40). This result parallels the finding in humans with presenilin 1 and presenilin 2 mutations showing more significant elevations of Aβ(1–42/43) than of Aβ(1–40) in serum and cultured fibroblasts (*31*). Studies correlating individual performance in learning and memory tests with concentration of Aβ and extent of amyloid deposition may help to ascertain the contribution of each parameter to behavioral deficits. Whether the learning and memory deficits in these mice are caused by or merely correlate with a rise in brain Aβ levels and amyloid deposition remains unresolved.

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18. The water maze was a circular pool (diameter 1 m) filled with water maintained at 29°C and made opaque by the addition of powdered milk. Mice were pretrained by swimming to a 12.7 cm by 12.7 cm Plexiglas platform that was submerged 1.5 cm beneath the surface of the water and placed at random locations within the pool. During pretraining, heavy curtains were drawn around the pool so that mice were unfamiliar with the extramaze room cues on the first day of spatial training. Spatial training consisted of four trials per day, each trial lasting until the mouse reached the platform or 60 s, whichever came first. After each trial, mice remained on the platform for 30 s. Twenty-four hours after the 12th and 24th trials, all mice were subjected to a probe trial in which they swam for 60 s in the pool with the platform removed. Mice were monitored by a camera mounted in the ceiling directly above the pool, and all trials were stored on videotape for subsequent analysis of platform crossings and percent time spent in each quadrant during probe trials. Visible-platform training—in the same pool but with a platform that was black, slightly larger (14.2 cm by 14.2 cm), and raised above the surface of the water—was given at least 24 hours after the second probe trial. The platform location was varied randomly from trial to trial to eliminate the potentially confounding contribution of extramaze spatial cues. In both visible-platform and hidden-platform versions, mice were placed in the pool facing toward the wall of the pool in one of seven randomly selected locations. The numbers of mice tested in the water maze were 12 transgenic and 12 controls at 2 months, 13 transgenic and 14 controls at 6 months, and 9 transgenic and 10 controls at 9 to 10 months of age.
19. The escape latency data were examined with a multifactor analysis of variance (ANOVA) including genotype (transgenic vs. control), age (2 months, 6 months, or 9 to 10 months), and training day (four trials per day). The ANOVA revealed significant main effects of genotype [$F(1, 384) = 65.19$, $P < 0.0001$], age [$F(2, 384) = 7.64$, $P < 0.001$], and trial block [$F(5, 384) = 12.20$, $P < 0.0001$]. Moreover, there was a significant interaction between genotype and age [$F(2, 384) = 10.13$, $P < 0.0001$], indicating that the transgene-induced impairment of escape latency increases with age.
20. All mice were also given a probe trial after 12 training trials (3 days at four trials per day). However, neither the transgenic nor the control mice had learned to search selectively after only 12 trials. The early probe trial was necessary because of the possibility of transient differences manifested only early in training, and because of the likelihood that we would have missed these differences because all behavioral tests were conducted blind to genotype. As none of the mice learned the task, there were no differences among any groups; for the sake of clarity, these data have not been presented graphically.
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32. We thank J. Loh, A. Mariash, J. Meiners, W. Yunis, H. B. Clark, D. Borchelt, G. Carlson, and T. C. Saido for advice and technical help. Supported by NIH grants NS33249 (K.H.), AG9009 (G.C.), AG06656 (S.Y.), and AG12685 (S.Y.), NSF grant IBN9410131 (P.C.), the Alzheimer's Association (K.H. and S.Y.), the California State Department of Health (G.C.), the American Health Assistance Foundation (S.Y.), and the Neurosciences Education and Research Foundation (K.H.). Care of experimental animals described was in accordance with institutional guidelines.

21 May 1996; accepted 5 August 1996

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2095–2097, March 1997

Commentary

The Alzheimer family of diseases: Many etiologies, one pathogenesis?

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The paper by Tomita and colleagues (1) completes a series of experiments carried out in many laboratories (2–4), which all lead to the same conclusion. Mutations in all three of the known pathogenic genes, the amyloid precursor protein (APP) gene (5), the presenilin 1 (PS1) gene (6), and the presenilin 2 (PS2) gene (7), have in common the fact that they alter APP processing such that an increased amount of the 42(43) amino acid peptide Aβ42(43) is produced (1–4, 8). The effects of the APP mutations (summarized in Table 1) have been elucidated over the past 5 years. However, the data implicating a link between the presenilins (PS) and APP began with the seminal observation that serum from individuals with PS-encoded Alzheimer disease (AD) contained, and that fibroblasts from such individuals produced, more Aβ42(43) (8). Since these observations were made in tissues from affected individuals, the effects of mutant PS on APP processing have been reproduced in a remarkably diverse series of tissues, in a large number of independent laboratories. Thus the effects have been seen in a range of transfected cells and in a variety of transgenic mice (some containing only the endogenous APP, and others doubly transgenic for both APP and PS wild-type and mutations) (1–4).

What are the implications of these data, and what questions now need to be addressed?

Perhaps the most obvious and important implication is that these data provide the strongest possible evidence that the "amyloid cascade hypothesis" for the etiology and pathogenesis of AD is correct, at least in outline (9, 10). While there have been a large number of hypotheses concerning the pathogenesis of this tragic and prevalent disorder, the genetic evidence, together with work such as that described by Tomita and colleagues (1), and pathological analyses showing that Aβ42(43) is deposited early and selectively in the plaques that are characteristic of the disease (11) and *in vitro* data showing that this form of Aβ is particularly fibrillogenic (12), all combine to give a single, coherent picture of the pathogenesis of the disease. In this scheme, Aβ deposition, precipitated by Aβ42(43) initiates the disease pathogenesis. All the other features of the disease: the neurofibrillary tangles, the synapse and cell loss, and the dementia follow from this critical initiating event. While the mutations in these genes account for only a small proportion of the total number of cases of AD, it is impressive that all known causes of AD, including Down syndrome, appear to share this common mechanism. The available data on AD in general would suggest that similar increases in Aβ42(43) may occur in a proportion of other, more "typical" AD cases (8): however, other mechanisms (such as decreased clearance of Aβ deposits) also may play a pathogenic role in some cases. This is important, not only from a purely scientific perspective, but also because it leads to the suggestion that therapies aimed at or downstream of APP processing have every chance of having general applicability. Of course, the existence of transgenic animals that replicate some of the features of the disease and constructed based on these genetic findings offers the potential to test these therapies in a comparatively rapid and efficient manner (13, 14).

However, the nature of the connection between the PS and APP processing remains obscure, and it is of importance to try and define the biochemistry of this relationship, not only for purely scientific reasons, but also because it may offer some novel therapeutic targets.

The first question relates to the nature of the effects of the PS mutations: do they represent a gain in function, a loss of function, or a gain of a novel function? Because all but one of the mutations have been missense mutations (15) [the single exception being an in-frame deletion of part of the hydrophilic loop of PS1 (see Fig. 1) (16)], the consensus was that these mutations most probably caused gains of misfunction (see, for example, ref. 15). This supposition was strengthened by the fact that mice expressing mutant PS genes (but still with their endogenous PS genes) showed the Aβ phenotype, suggesting that normal levels of the wild-type endogenous protein could not compensate for the mutant (2–4). However, more recent work, particularly on the *Caenorhabditis elegans* isologues of the PS, has cast doubt on this explanation.

Two *C. elegans* isologues of the PS have been identified: *spe-4* and *sel-12* (17, 18). Both are recessive phenotypes. *spe-4* mutants have a defect in spermatogenesis believed to be caused by a problem with protein trafficking in the Golgi (17), and *sel-12* mutants have an egg-laying defect in which the notch pathway is implicated (18). Molecular analysis of the latter with respect to the PS has been particularly informative, because, in this pathway, wild-type PS rescues the phenotype, but several missense mutations do not (ref. 19, †). Also, work in which PS expression is reduced in cells transfected with antisense constructs has shown that this reduction of PS expression leads to an increase in Aβ42(43) production.‡ These data strongly suggest that there is a loss of function component to the effects of the mutations (19), although it is clear that complete loss of function is extremely unlikely because mice in which the PS1 gene has been knocked out, die *in utero*.§ If loss of PS function is critical to the AD phenotype, then understanding the normal roles of this family of proteins becomes key. To date, we have only the sketchiest of ideas as to what these roles might be. Clearly, further genetic analysis of *C. elegans* is warranted, specifically to determine other components of the *spe-4* and *sel-12* pathways so that their human homologues can then be tested for any role in the expression of the Aβ phenotype. Thus, it may well be that assessment of Aβ42(43) will be used as a surrogate marker for AD, and that egg-laying defects in *C. elegans* will be used as a

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†Haass, C. & Baumeister, R., Data presented at Society for Neuroscience Meeting, Washington, DC, November 1996.

‡Younkin, S. & Refolo, L., Data presented at Society for Neuroscience Meeting, Washington, DC, November 1996.

§Sisodia, S. & Wong, C., Data presented at Society for Neuroscience Meeting, Washington, DC, November 1996.

0027-8424/97/942095-3$2.00/0
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Table 1. The known routes to Alzheimer disease

Primary cause	Route	Final result
Down syndrome	More APP production	More Aβ42(43) and more Aβ40
$APP_{670/1}$ (Swedish)	Potentiation of β-secretase	More Aβ42(43) and more Aβ40
APP_{692} (Flemish)	Inhibition of α-secretase	More Aβ42(43)
APP_{717} (London)	Alteration of site of γ-secretase cut	More Aβ42(43)
PS1 mutations	Subtle alteration of APP processing	More Aβ42(43)
PS2 mutations	Subtle alteration of APP processing	More Aβ42(43)

surrogate for the Aβ phenotype. The development of powerful molecular screens, such as these, for identifying components of the early stages in the pathogenesis of the disease, can only speed the process of understanding the disease.

The cliché that many reviews (and grant applications) on AD used to start with was "Alzheimer disease is a dementia of unknown cause and inexorable progression." Over this last couple of years, this cliché has become no longer true. We now know several causes of the disease (all genetic so far), and all of these "causes" have pointed toward the path of APP metabolism and Aβ production and deposition as being the key early event in disease pathogenesis. Unfortunately, the "inexorable progression" part of the cliché remains true; however, genetics and molecular biology now are revealing credible drug targets, and it is to be hoped that relatively soon we will be able to turn this leap in understanding into effective therapy. The unofficial "goal" of the National Institute on Aging was to have some form of effective therapy by the year 2000. This ambitious goal may yet be realized.

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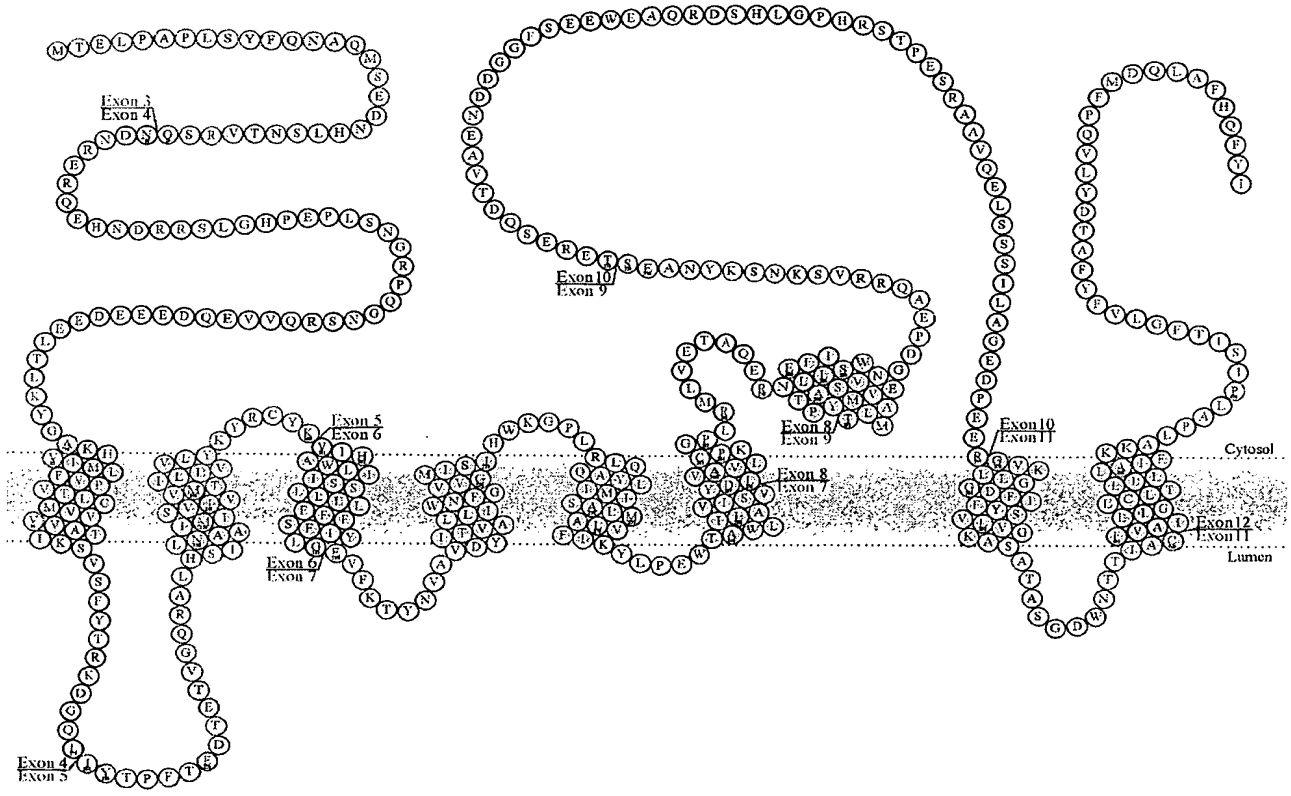

FIG. 1. The structure of the PS1 protein derived from the author's interpretation of ref. 20, showing the positions of the exon boundaries (21) and mutations (reviewed in ref. 22).

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PHYSIOLOGICAL REVIEWS
Vol. 78, No. 4, October 1998
Printed in U.S.A.

Using Knockout and Transgenic Mice to Study Neurophysiology and Behavior

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Picciotto, Marina R., and Kevin Wickman. Using Knockout and Transgenic Mice to Study Neurophysiology and Behavior. *Physiol. Rev.* 78: 1131–1163, 1998.—Reverse genetics, in which detailed knowledge of a gene of interest permits in vivo modification of its expression or function, provides a powerful method for examining the physiological relevance of any protein. Transgenic and knockout mouse models are particularly useful for studies of complex neurobiological problems. The primary aims of this review are to familiarize the nonspecialist with the techniques and limitations of mouse mutagenesis, to describe new technologies that may overcome these limitations, and to illustrate, using representative examples from the literature, some of the ways in which genetically altered mice have been used to analyze central nervous system function. The goal is to provide the information necessary to evaluate critically studies in which mutant mice have been used to study neurobiological problems.

I. INTRODUCTION

One fundamental goal of biological science is to determine the physiological function of identified proteins. This is a daunting task, particularly since a given protein functions within the context of thousands of other proteins found in every cell. For proteins found in the brain, the center of such complex activity as cognition, emotion, and learning, the task is even more daunting. One approach is to remove the protein from its normal environment and study it in a more simple setting, such as a reconstituted in vitro system. Although inferences with respect to normal physiological role can, and have, been made from such studies, conclusions are necessarily restricted. In vitro and heterologous expression systems are useful precisely because they reduce the complexity of the living cell. Nature has provided some clues, in the form of genetic diseases, to the physiological relevance of certain proteins. Linkage of particular gene defects to genetic diseases has greatly enhanced our understanding of the role of many proteins. Indeed, studies of naturally occurring gene mutations leading to neurophysiological defects in mice and humans have enhanced our understanding of several proteins found in the central nervous system (CNS) (for reviews, see Refs. 11, 57, 203, 237). More recently, technology has evolved that allows the specific modification of the genetic composition of many organisms. This type of reverse genetic approach, in which detailed knowledge of the gene of interest permits in vivo modification of its expression or function, provides a powerful method for examining the physiological relevance of any protein.

When studying proteins involved in biochemical or developmental pathways, simple cellular processes, or behaviors conserved throughout evolution, the classical subjects of genetic research such as yeast, *Drosophila melanogaster*, and *Caenorhabditis elegans* are superior to the mouse from the standpoints of ease of genetic manipula-

tion, the number of organisms that can be generated and studied, and the cost and time investment. Unique features of yeast make disruption of specific genes relatively simple, while studies with *Drosophila* and *C. elegans* are not restricted by the relatively slow time course of mouse studies. Indeed, the 3-wk gestation period, subsequent 2 mo to reach sexual maturity, and comparably small litter size (usually 2–10 pups) make mouse studies time and space consuming, and therefore costly. Mice possess a distinct advantage over classical genetic subjects, however, in that mice are more closely related to humans from a physiological perspective. Although some genetic studies have been performed using rats (for review, see Ref. 37), the vast majority of transgenic research has used mice, primarily because of the greater technical ease of manipulating the mouse embryo, the lack of classical genetic information (genetic locus markers), and the smaller number of inbred rat strains. In addition, rats lacking a specific gene of interest have not been generated, since it has not been possible to date to create pluripotent rat embryonic stem cell lines that are necessary for this technique (see sect. III*C* for discussion of embryonic stem cells). Thus, when studying the role of a protein in a complex behavior such as learning, or when trying to model a human disease, mice are often the research subjects of choice.

Complex behaviors are determined by the function of many gene products. Thus some of the same features that make mouse models advantageous for studying the molecular basis of complex behaviors can make the interpretation of phenotypes resulting from mouse mutagenesis difficult. For example, several variables, including the expression level and allelic variants of other genes, can influence phenotypes revealed by physiological studies. Thus useful information from studies of genetically altered mice can only be obtained if appropriate controls are employed and data are interpreted within the context of the experimental limitations.

Genetically altered mice have been used in the fields of immunology and developmental biology with great success for more than a decade. In the last 5 years or so, we have seen increased use of mutant mice to study the complex functions of the CNS. One goal of this review is to illustrate, using representative examples from the literature, some of the ways in which genetically altered mice have been used to analyze CNS function. Because of the logarithmic expansion in mouse genetic studies involving molecules important in the CNS, this review cannot be comprehensive. A second goal of this review is to familiarize the nonspecialist with the techniques and limitations of mouse mutagenesis and to describe new technologies that may overcome these limitations. Interested readers are also directed to recent excellent articles that discuss specific techniques (37, 196, 264, 282) or review in a more comprehensive manner the data that have been obtained from mutant mice (6, 40, 55, 286).

II. WHY CREATE A MUTANT MOUSE?

Genetically altered mice are used increasingly as tools to define or clarify the in vivo function of molecules that have been studied in vitro. The phenotypes of a mutant mouse can be studied at many levels from biochemistry to cell biology to systems physiology to behavior. One of the advantages of a mutant mouse that survives to adulthood is that the effect of a single gene alteration on a complex behavior can be studied. For example, the role of many signal transduction pathways in learning and memory has been investigated using mutant mice.

In addition to being a tool for understanding the physiological function of particular proteins, mutant mice can also be used to model human diseases. As the genes responsible for human genetic diseases are identified through linkage studies, it becomes feasible to create mouse models of those diseases (reviewed in Refs. 5, 27, 52, 156, 164, 218). Current technology allows modeling of both gain-of-function disorders (in which a mutant protein with altered function is expressed or a wild-type gene is overexpressed) and loss-of-function disorders (in which an endogenous gene is inactivated or a mutant protein is nonfunctional). The hope in these studies is that the altered mice will exhibit symptoms similar to a human patient and that the pathogenesis of the disease is similar between the species. There are several advantages to a mouse model of human disease, including the possibility of studying the gene mutation in multiple individuals with a homogeneous genetic background, the ability to study disease onset and progression in a highly controlled environment, and the ability to study early stages of a disease before the appearance of overt symptoms. Currently, mice are being used to model several human neurological diseases, including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and transmissible spongiform encephalopathies (prion diseases). Although no perfect model of any of these disorders exists, invaluable information regarding disease mechanisms has already been gained from these attempts.

Several other benefits can come from mutant mouse studies. Assumptions about the in vivo targets of particular drugs can be tested rigorously using genetically altered mice. By ablating a single protein or subunit of a complex, one can test whether the protein is the target of a commonly used agonist or antagonist. For example, the γ_2-subunit of the $GABA_A$ receptor was thought to be a critical site of action for benzodiazepines, widely used anxiolytic agents (217). This hypothesis was confirmed in mice lacking the γ_2-subunit, which had a 94% reduction in benzodiazepine binding sites in the brain (98). Similarly, the α_6-subunit of the $GABA_A$ receptor was thought to mediate the behavioral effects of ethanol (163); mice lacking the α_6-subunit, however, exhibited unimpaired ethanol-induced sleep (112), demonstrating that at least one aspect

of ethanol function is not mediated through this subunit. When typical pharmacological tools are unavailable, mutant proteins that ablate the function of their wild-type counterparts (dominant negative mutants) can be used as in vivo antagonists. For example, a dominant negative form of the cAMP-responsive transcription factor CREB was expressed in mice by transgenesis to evaluate the role of CREB in the transcription of the tyrosine hydroxylase (TH) gene (154). Overexpression of the dominant negative form of CREB inhibited TH transcription, implying that endogenous CREB, or another CREB family member, normally drives this process.

III. GENERATION OF MUTANT MICE

For those not intimately involved in the generation and study of mutant mice, it can be difficult to evaluate such studies critically. In this section, we describe the theory, methodology, and limitations involved in the generation of mice expressing (transgenic) or lacking (knockout) a particular gene of interest.

A. Transgenic Mice

The creation of a transgenic mouse begins with the selection of a transgene, the DNA element to be transcribed, and usually translated, in the mouse. The element is often a cDNA encoding all or part of a particular protein, but it can be a genomic fragment containing all or part of a gene, or an antisense fragment designed to deplete the level of an endogenous mRNA (for examples of transgenic antisense studies, consult Refs. 82, 192, 193). The transgene is subcloned downstream of a suitable promoter element that will drive its expression. To a large extent, the promoter element determines the level, tissue specificity, and temporal pattern of transgene expression. For studies in the nervous system, transgene expression can be controlled by characterized minimal promoter sequences that target the gene to specific cells or brain regions, such as the glial fibrillary acidic protein (GFAP) (25) or myelin basic protein (94) promoters (glia), neuron specific enolase (76) or calmodulin II (177) promoters (neurons), calcium/calmodulin-dependent kinase II (CaMKII)-α promoter (forebrain/hippocampus; Refs. 181, 278), or by elements providing a more ubiquitous neuronal expression pattern such as the β-actin promoter (134). If a close approximation of the expression pattern of the endogenous gene is desired, as is the case in most disease-modeling experiments, one can use the gene's own promoter. The temporal pattern of expression of the transgene can also be affected by its promoter. Indeed, some studies have utilized promoters that turn on after birth to avoid potential developmental effects of chronic transgene expression during prenatal development (181).

The transgene is introduced into single-cell mouse embryos by pronuclear injection (Fig. 1; for an extensive review of practical methods, consult Ref. 111). Once inside the pronucleus, the transgene either integrates into the genome in a random fashion or is degraded by exonuclease activity. Those embryos that integrate the transgene usually acquire several copies in a tandem head-to-tail array. Transgene insertion usually occurs quite early in the developmental process; thus most or all of the cells that comprise the resultant mouse contain the transgene. However, if insertion occurs after several rounds of cell division, the transgene may be present in only a subset of cells. The resulting mouse will be mosaic, with only a subset of cells containing the transgene. If the transgene is present in cells comprising the germ line, it will be transmitted to subsequent generations of mice. Mice that transmit the transgene through the germ line are termed founder mice, and although they can be examined for phenotypic consequences of transgene expression, founders are more often used to generate large numbers of transgenic animals. Occasionally, the transgene integrates into more than one site in the genome, and offspring from such a founder animal may inherit one or the other copy of the transgene, resulting in multiple lines with potentially distinct phenotypes from a single founder (278).

Not all founder mice are alike, despite the fact that inbred mouse lines, in which all mice are genetically identical, are often the source of the embryos. One key difference between founders is the number of copies of the transgene integrated into the genome. In fact, founder mice vary widely in the number of transgene copies, usually between 2 and 50, that are integrated into the genome. The expression level of the transgene is often positively correlated with its copy number. In some cases, then, mice containing different numbers of transgene copies can exhibit distinct phenotypes. For example, an attempt to model familial ALS using a single transgene yielded several mouse lines that all exhibited progressive muscle wasting leading to death, but the disease progression rate was generally proportional to the transgene copy number, and histopathological features observed in certain lines were not observed in others (54).

Although copy number can affect the level of transcription of the transgene, a more important distinction between founder mice often involves the site of integration of the transgene within the genome. Most transgenic studies show extensive positional effects, including undesirable expression of the transgene in a particular tissue(s) (ectopic expression). The level and spatial distribution of transgene expression are sensitive to its proximity to transcriptional activators or silencers in the genome. If the transgene of interest is expressed under the control of a neuron-specific promoter, but inserts into the genome near a liver-specific enhancer, the transgene could be ex-

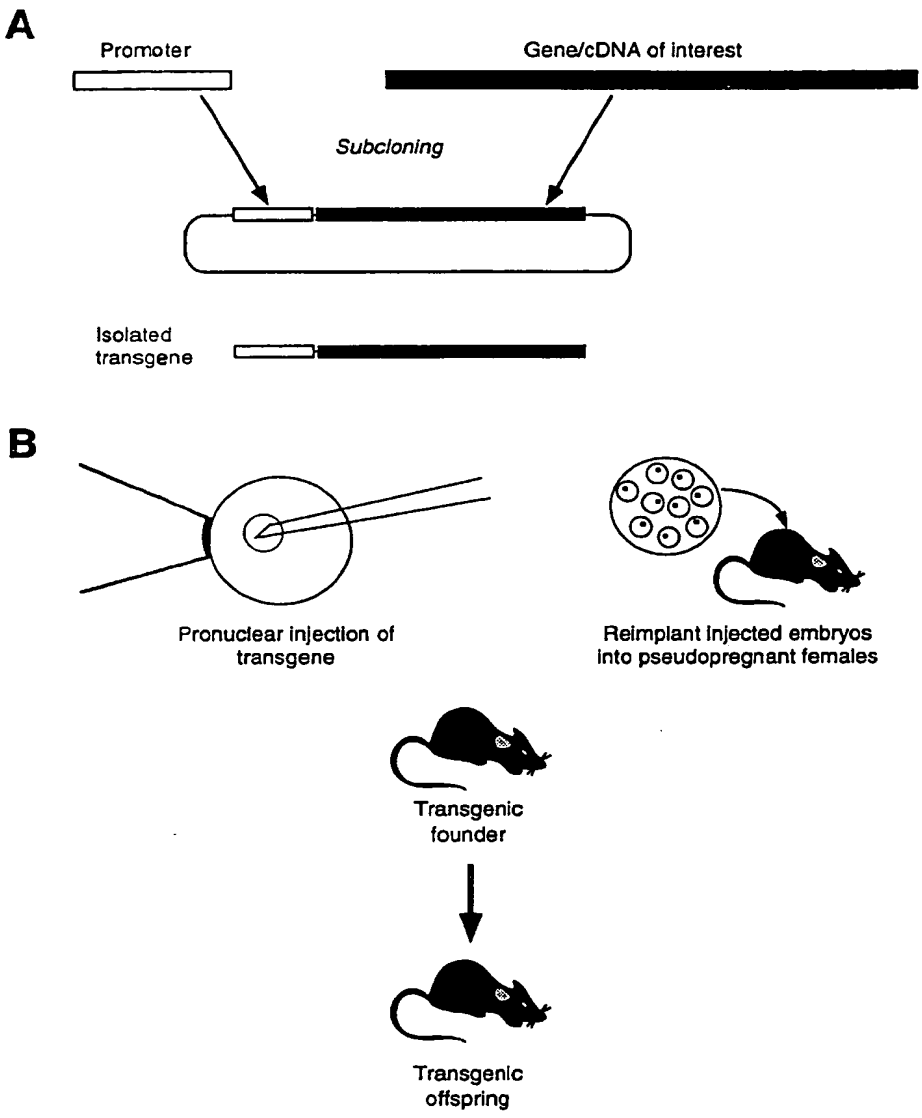

FIG. 1. Classical transgenic construction. *A*: a segment of DNA to be transcribed (cDNA, genomic fragment, antisense fragment) is subcloned downstream of a neuron-specific promoter (e.g., neuron-specific enolase). *B*: after separating transgene from plasmid sequence, isolated fragment is injected into pronucleus of a single-cell embryo. Ten to twenty injected embryos are implanted into a foster mother. Some mice (transgenic founders) carry transgene in all cells including germ line (eggs or sperm) and thus can transmit gene to subsequent generations. Gene of interest should be expressed predominantly in brain, although insertion effects may result in ectopic expression.

pressed both in neurons and in the liver. Thus ectopic, inappropriate, or unintended expression of the transgene can lead to phenotypes that nonspecifically affect the nature of the system to be studied. Silencer elements are also distributed throughout the genome, making it possible that some integration sites yield little or no expression of the transgene. To control for positional influence when screening for phenotypes, it is necessary to generate a number of founder mice and compare phenotypes among their offspring.

Just as the genome can influence transgene expression, the transgene can alter the expression of neighboring genes. Although only a small percentage of the genome consists of actual protein coding sequence, genes often span several hundred kilobases of DNA. Therefore, transgene integration can disrupt protein coding sequence, promoter elements, or other less-understood regulatory elements controlling gene expression. In one case, insertion of an interferon transgene disrupted the monoamine oxidase A gene, resulting in hyperaggressive mice with high monoamine levels due to the disruption rather than the overexpression of interferon (34). Random transgene integration has been exploited by other groups to purposely disrupt, and consequently mark, genes that can be cloned later by screening of a library constructed from genomic DNA (61, 78).

Since positional effects related to transgene integration site were first appreciated, attempts to minimize inconsistency in phenotypes between founder mice have been made. One approach to combat ectopic expression and positional effects has been to create mice using transgenes consisting of large (>50 kb) genomic fragments containing intact genes and regulatory elements with desired alterations. Unfortunately, genomic fragments of this size are not easily manipulated or mutated in classical bacterial plasmids or cosmids. In contrast, yeast artificial chromosomes (YAC) are useful cloning vectors that can accommodate very large genomic fragments (up to 2 megabases) that can be modified efficiently in yeast by homologous recombination (213). In brief, a small plasmid construct carrying a portion of the large genomic fragment with the desired alteration, such as a cDNA to be used as a transgene, plus a selectable marker, is introduced into yeast (Fig. 2). The fragment carrying the desired mutation then recombines or exchanges with the homologous portion of the large fragment in the YAC, transferring the alteration to the large fragment (23, 269). Although YAC have been used successfully as transgenes in mouse experiments, several features of this system have limited its utility. First, isolation of intact YAC and introduction into the germline by classical pronuclear injection are difficult because of the unavoidable shearing stress and viscosity of these large fragments. Although more gentle alternatives to the pronuclear injection of YAC into mouse embryos have been developed, YAC also exhibit a high degree of chimerism and clonal instability (189). In other words, a YAC may acquire deleterious or unwanted mutations when passaged in yeast. Bacterial artificial chromosomes (BAC, based on the *Escherichia coli* fertility factor) and P1-derived artificial chromosomes (PAC, based on bacteriophage P1) are cloning vectors that propagate genomic fragments up to ~300 kb in *E. coli* and have several advantages over YAC. First, BAC and PAC are stable and exhibit minimal chimerism when passaged in recombination-deficient bacteria (189). Second, purification of PAC and BAC is relatively easy since they exist as supercoiled circular plasmids that are resistant to shearing, making them amenable to pronuclear injection. Third, as was recently demonstrated, BAC can be modified by homologous recombination and utilized as standard transgenes (296). Thus YAC, BAC, and PAC technologies could dramatically improve the quality of mouse transgenesis in the future.

B. Inducible Expression of a Transgene

Several genes of interest for neurobiological research, such as protein kinases, transcription factors, and growth factors, subserve critical functions throughout development. Chronic expression of a transgene could therefore cause a developmental abnormality or adaptation, leading to masking or distortion of the acute role of the protein of interest. To avoid the complication of functional or developmental compensation or drastic developmental phenotypes, temporal control over transgene expression is desirable.

Several inducible systems are available that are based on a common theme (238). These inducible systems are exemplified by the tetracycline (Tet)-regulatory system (Fig. 3; Refs. 79, 92, 137, 253). In this system, the generation and crossing of two different lines of mice are required. The first mouse line expresses the Tet transactivator (tTA) under the control of a promoter that directs its expression in the tissue or tissues of interest. Tetracycline transactivator is a transcription factor that regulates the expression of any gene downstream of its cognate promoter sequence (TetOp). The second mouse line harbors the transgene consisting of the gene of interest downstream of TetOp. Inducibility in the Tet system depends on the presence of Tet or a Tet analog such as doxycycline (Dox). In the "Tet-off system," Tet inhibits the interaction between tTA and TetOp, effectively suppressing transcription of the gene of interest.

One disadvantage of the Tet-off system is that induction of the transgene only in the adult animal requires the persistent exposure of mice (beginning at conception) to levels of Tet or Dox sufficient to prevent transgene expression. This can be expensive and may result in unknown behavioral side effects. Indeed, exposure to Dox during development in one study resulted in adult mice with impaired spatial memory and fear conditioning (180). Furthermore, the rate at which induction can occur is limited by the clearance of Tet or Dox from the animal, and this rate varies from tissue to tissue (137). To maximize responsiveness and minimize the side effects of the antibiotics, mice are usually exposed to the minimum amount of Tet or Dox required to repress transgene transcription.

An alternative system that eliminates the necessity for chronic exposure to Tet or Dox utilizes a mutant form of tTA, reverse tTA (rtTA), which is activated rather than repressed by Tet or Dox (93). Thus the expression of the gene of interest occurs in the presence of Tet or Dox ("Tet-on"). Because transgene expression does not depend on the clearance of antibiotic from the tissue of interest, this system might offer more rapid induction. This system was used in transgenic mice, and induction over several orders of magnitude was achieved in 4 h in some tissues and was usually complete after 24 h (137). This study reported strict dependence of transgene expression on the presence of antibiotic, but a high degree of basal expression (leakiness) has been seen in cell culture using this system (Picciotto, unpublished data).

The Tet-off system has been used to direct transgene expression in specific brain regions at particular times in adult mice (180). The CaMKII-α promoter was used to

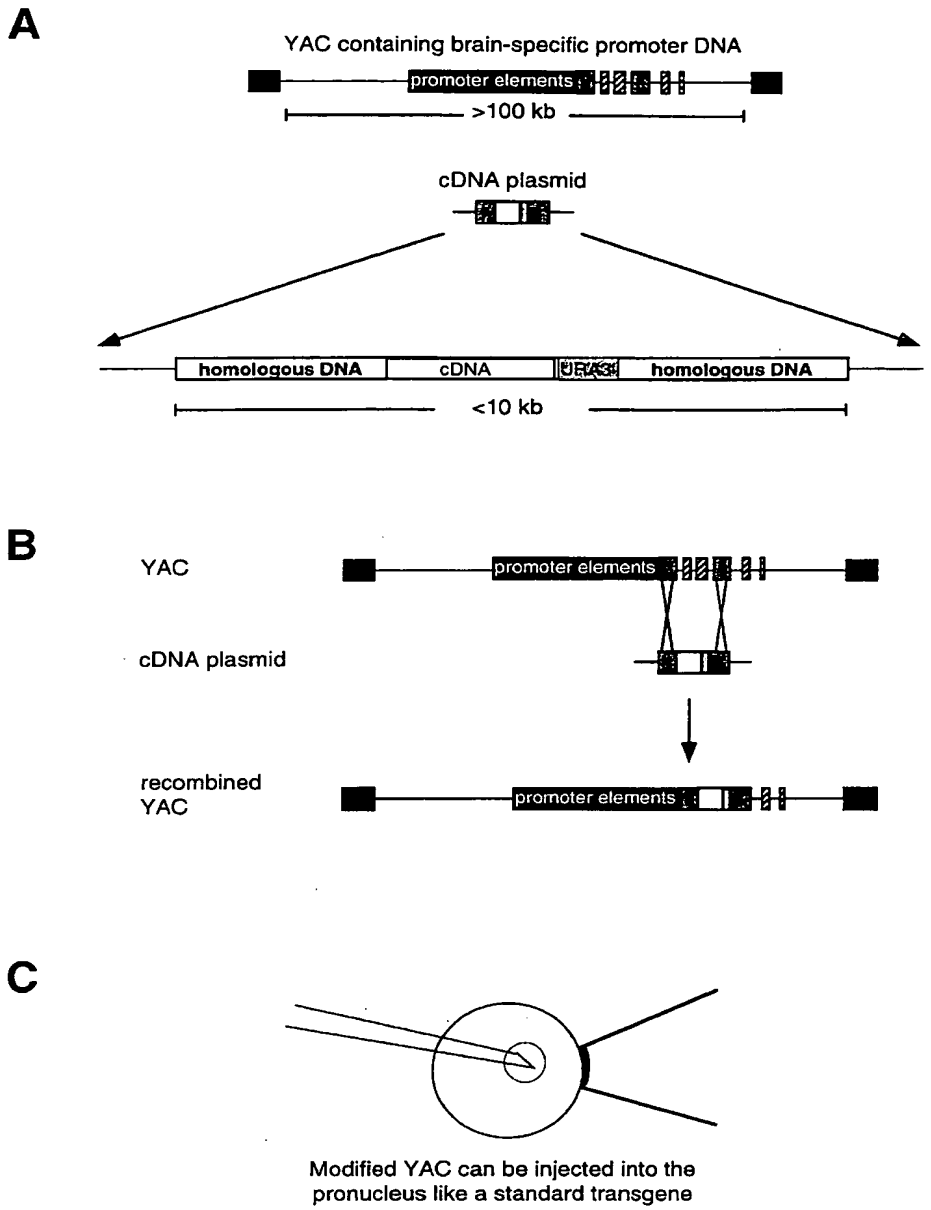

FIG. 2. Use of yeast artificial chromosomes (YAC) as transgenes. Very large fragments of genomic DNA can be manipulated using YAC as vectors. In this example, promoter sequences contained within a large fragment of DNA can be used to drive expression of a cDNA of interest. *A*: first, a YAC is identified containing desired promoter. A plasmid is then constructed containing cDNA of interest flanked by short segments of genomic DNA homologous to site at which transgene will be inserted (in this example, insertion site would be immediately after promoter elements). This transgene plasmid would also contain a selectable marker such as *URA3* gene that allows yeast to grow in absence of uracil in medium. *B*: cDNA-containing plasmid is then introduced into yeast carrying YAC, allowing recombination to occur between cDNA-containing vector and YAC. In this example, cDNA is inserted downstream of large promoter fragment in position of endogenous gene. cDNA-containing plasmid itself does not replicate in yeast; therefore, only those yeast that have undergone recombination with plasmid, and incorporated *URA3* marker, will survive when plated on medium lacking uracil. *C*: recombined YAC can then be isolated and injected into oocytes like a standard transgene. This technique could also be used to express a mutated transgene under its own promoter.

restrict tTA expression to the forebrain. These mice were then crossed with another transgenic line harboring a mutated (constitutively active) form of CaMKII-α under the control of TetOp, to generate bigenic mice (mice containing both transgenes). In the presence of Dox, which penetrates the blood-brain barrier and regulates tTA activity more effectively than Tet, transcription of the mutant CaMKII transgene in the brain was repressed. When Dox was removed, the mutant kinase was expressed in different subregions of the forebrain, depending on the mouse line used. Interestingly, induction of the mutant kinase in the hippocampus throughout development altered their spatial learning capabilities and the electrophysiological responses of hippocampal neurons to changes in the environment, and this effect was reversed by treatment with Dox in the adult.

Other inducible systems based on the triggering substances interferon-γ (144) and glucocorticoids (135) have been reported, but the associated nonspecific effects of these drugs are pronounced in mice and could induce

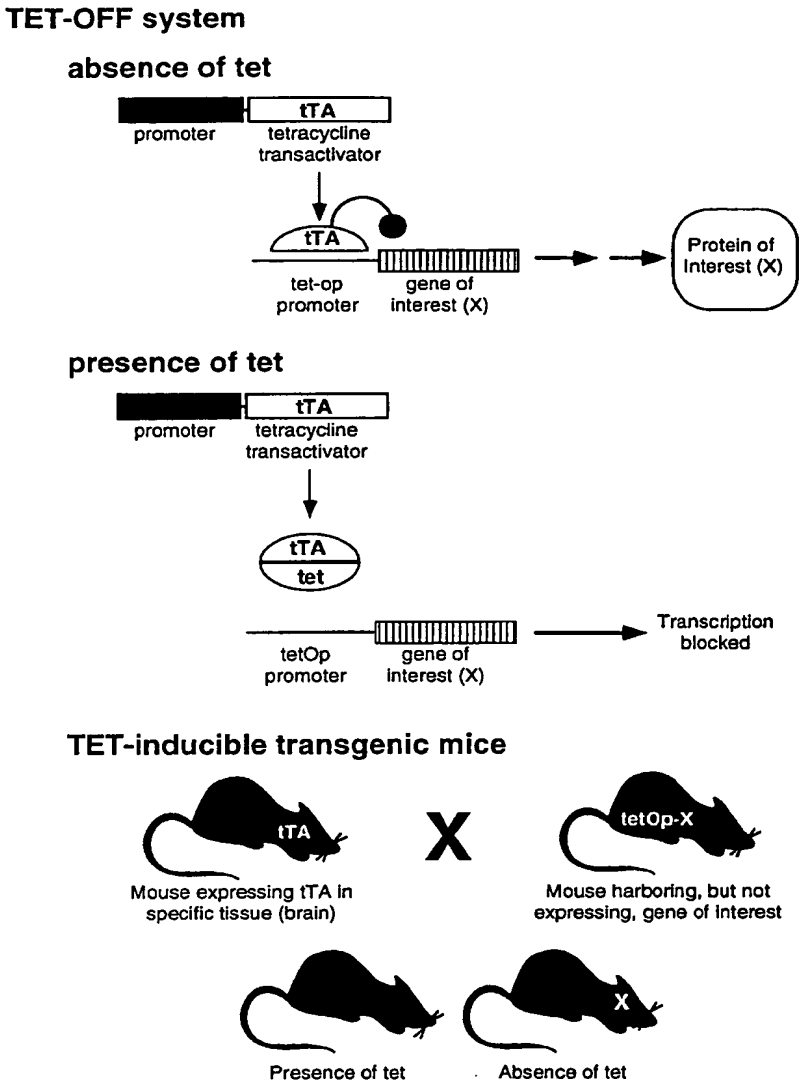

FIG. 3. Inducible transgene expression. In tetracycline (Tet)-off system, transcription of gene of interest (X) occurs when Tet transactivator (tTA) binds to TetOp promoter. In presence of Tet, tTA is blocked from activating transcription. System can be introduced into mice by creating 2 independent transgenic mouse lines. First mouse expresses tTA in a specific tissue (brain). Second line harbors gene of interest driven by TetOp promoter (TetOp-X). When lines are crossed, and in absence of Tet, those tissue(s) expressing tTA will also transcribe gene X. When tetracycline is present, however, transcription is blocked. tTA has also been modified so that gene transcription occurs only in presence of Tet (see text).

phenotypes unrelated to the transgene. A system based on induction of the progesterone receptor by the synthetic steroid hormone RU-486 has been used in mice to express a reporter gene (59), but once again, RU-486 has powerful effects on behavior and physiology on its own, and this could affect the interpretation of the transgenic phenotype. Another system involves the insect molting hormone ecdysone as an inducer that does not have a target in mammalian cells. Recently, a transgenic mouse line expressing a modified ecdysone receptor dimer was generated and was bred with a second mouse harboring a transgene consisting of the ecdysone responsive promoter and reporter gene (205). Induction reaching four orders of magnitude was achieved with low or undetectable leakage transcription. One disadvantage of this system is that three different transgenes must be present in the cell type of interest. The chances of obtaining mice expressing the receptor subunits in a uniform and overlapping fashion are therefore low, breeding strategies become complex, and controlling for integration site effects is difficult.

The critical variables that differ between existing inducible systems include the amount of transcription that occurs when transgene expression is intended to be off (leakage transcription), the extent to which expression can be increased (dynamic range), the speed with which transgene expression can be up- or downregulated (pharmacokinetics), and side effects related to the exposure of the mouse to the triggering substance. Available systems

each have strengths and weaknesses that need to be addressed in the context of the experimental question to be answered.

C. Gene Knockout Through Gene Targeting

A useful method to determine whether a gene product of interest is necessary for mediating a particular process is to abolish its expression and evaluate the consequences. Random gene inactivation can be accomplished using ultraviolet irradiation, mutagenic chemicals, viruses, or transgenes, all agents which randomly attack the genome. Coupled with in vivo selection assays based on the function of the gene product of interest, this approach can be used to clone novel genes, particularly in organisms with a short generation time such as *C. elegans* and *D. melanogaster*. In contrast to random mutagenesis, targeting via homologous recombination permits directed genomic lesions that either abolish gene expression or alter the gene of interest (for excellent reviews, see Refs. 32, 264, 284). As the phrase suggests, homologous recombination is the in vivo exchange of genomic sequence between homologous fragments of DNA. In gene targeting, two genomic fragments that closely match or are identical to regions in the genomic locus to be targeted flank a selectable marker (making up the targeting vector or targeting construct) (Fig. 4). The targeting construct is introduced into host cells, and when homologous recombination occurs, a part of the genomic DNA of interest will be replaced with DNA found between the homologous flanking domains in the targeting construct. Depending on the design of the targeting construct, several variants of the technique are possible, allowing deletions, point mutations, or replacements (264).

From a practical standpoint, gene targeting in mice begins with the isolation and mapping of a mouse genomic fragment encoding all or part of the gene of interest. A combination of Southern blotting, sequencing, and/or PCR is used to determine the gene structure sufficiently to design a targeting strategy, as well as to develop a screening strategy to identify homologous recombination when it does occur. Usually, a strategy is developed so that an exon containing the initiator methionine of the gene of interest or a known functional domain is removed and replaced with a selectable marker such as the neomycin resistance (neo^r) gene driven by a ubiquitous promoter such as the murine PGK-1 promoter (4). Integration of the DNA into the host genome is thus selected for by incubation in neomycin-containing (geneticin, G418) growth medium (positive selection).

In mammalian cells, homologous recombination occurs at low frequency (33, 284). In the majority of transfected cells, then, the targeting construct inserts randomly. To enrich for cells that undergo homologous recombination, many targeting constructs contain a second selectable marker that flanks one of the homology domains (303). Because it is positioned outside of the area of genomic homology, it is spliced out when homologous recombination occurs and remains intact if the construct inserts randomly. In contrast to the positive selection marker, this marker kills the host cell when integrated as part of the targeting vector (negative selection). The most common negative selection markers are the herpes simplex virus thymidine kinase gene (272) and the diphtheria toxin gene (295).

Several factors influence the frequency of homologous recombination, or targeting efficiency, including the extent of identity between the construct and the genomic locus (303). Overall identity is dependent on the length of the genomic fragments (generally a total of at least 6 kb of homologous DNA) and the source of the fragments used to create the targeting construct. A positive correlation between the combined length of the homologous fragments and targeting efficiency has been observed (273). Targeting efficiency is also improved when the DNA fragments in the targeting vector are obtained by screening a genomic library made from the same strain of mouse that was used to derive the cells for the targeting experiment (271, 283). In addition, it seems that targeting efficiency is highly dependent on the targeted locus itself, probably because of factors such as the specific DNA sequence involved (103), extent of DNA methylation (161), or particular chromatin structure (219). Interestingly, the extent of the deletion created by the recombination event does not appear to reduce targeting efficiency (188).

Once made, the targeting construct is linearized and introduced into embryonic stem (ES) cells by electroporation (Fig. 4). The utility of gene targeting in mouse genetic studies was made possible by the isolation and culture of murine ES cells (21, 22), which are nontransformed cells derived from normal mouse embryos that have retained the ability to divide in culture but remain pluripotent. In other words, these cells are capable of differentiating into any tissue of the developing mouse. Embryonic stem cells have been isolated and cultured by several laboratories and are now commercially available. The vast majority of ES cells are derived from the 129 mouse strain, and thus the majority of gene targeting constructs are also prepared using genomic DNA from this mouse strain. However, it is likely that ES cells and genomic libraries from other strains will become increasingly available. The ability to culture ES cells makes them suitable for gene targeting experiments, whereas the pluripotency of ES cells makes it possible to derive mice from these cells, even after they have been cultured. The combination of these features forms the backbone of gene targeting experiments in mice.

After electroporation and antibiotic selection, a suit-

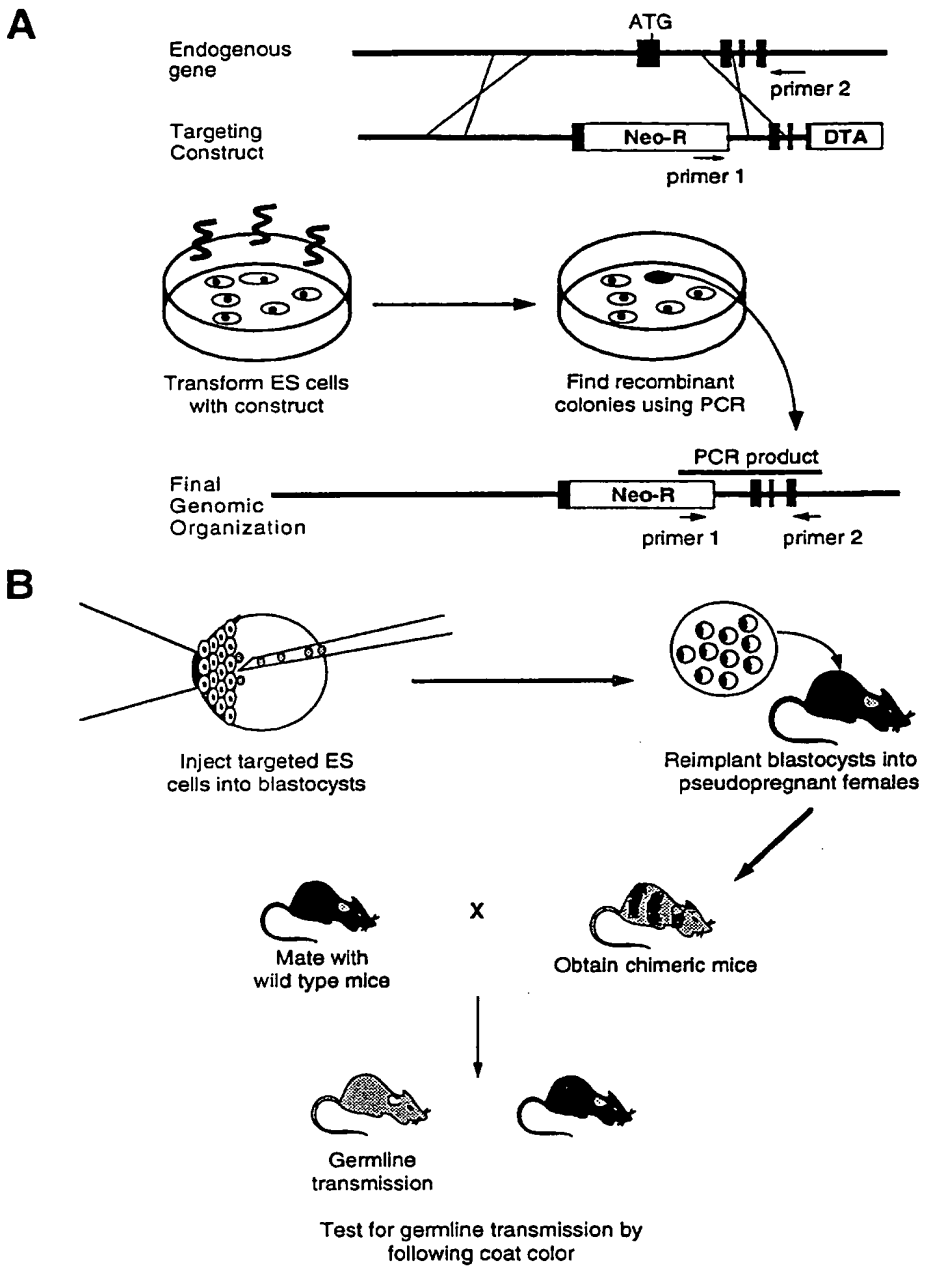

FIG. 4. Classical gene knockout. *A*: wild-type allele, targeting vector, and targeted allele following homologous recombination. Targeting construct is introduced into embryonic stem (ES) cells by electroporation, and subsequent selection in G418 eliminates all ES cells that do not acquire a copy of this construct. Generally, homologous recombination occurs infrequently compared with random integration of construct. To enrich for homologous recombinants, diphtheria toxin (DTA) or thymidine kinase genes can be included in targeting construct. Homologous recombination can be detected by PCR screening using primers just outside shortest domain of homology used to make targeting construct. Neo-R, neomycin resistant. *B*: when ES cell clones are identified with a targeted allele, 10–20 cells are injected into blastocysts (3.5-day embryos), and 10–20 blastocysts are reimplanted into each foster mother. In some cases, injected pluripotent ES cells contribute to a large percentage of resultant mouse tissues (chimeric mouse). If ES cells and blastocysts were derived from mouse strains with different coat colors, degree of contribution of ES cells to resultant mouse can be approximated by coat color. Targeted allele can be transferred to subsequent generations if chimeric animal's germ cells were derived from targeted ES cells. Germline transmission is also monitored by following coat color.

able number of colonies (generally between 50 and 500) are picked for DNA isolation and are screened by Southern blotting or PCR to detect ES cell colonies in which the DNA is correctly recombined. The method of screening dictates in part the design of the targeting construct (see, for example, Refs. 158, 215) as one arm of genomic DNA must be <2 kb long if PCR screening is to be used. After amplification of the colony, recombined ES cells are then microinjected into 3.5-day-old blastocysts, usually derived from the C57BL/6 mouse strain. After microinjection, blastocysts are reimplanted into the uterus of pseudopregnant females and are carried to term. Usually a fraction of the resulting mice are chimeric, that is, they have developed in part from the targeted ES cells and in part from cells of the donor blastocyst. Chimerism can be assessed visually if the ES cells and donor blastocysts are derived from

strains of mice with different coat color. Male chimeras judged to be derived to a large extent (50% or more) from ES cells are then crossed to female mice, and germline transmission is assessed by the coat color of the resultant offspring. Embryonic stem cell gene targeting usually yields cells carrying a single disrupted allele of the gene of interest; thus offspring of chimeras have a 50% probability of harboring a targeted allele. These mice are typically identified by PCR amplification of a portion of the targeted allele from tail biopsy DNA. Mice containing one wild-type and one targeted allele (heterozygotes) can then be intercrossed to generate mice homozygous for the targeted allele (knockouts). Generally, the targeted mutation is inherited in a classical Mendelian fashion.

Depending on the gene-targeting strategy, different phenotypes can be observed. For example, different approaches to inactivating cystic fibrosis transmembrane conductance regulator (CFTR), the gene linked to cystic fibrosis, led to different phenotypes in the resultant mice. One group removed a functional portion of the CFTR coding region and replaced it with neo^r (261). These mice died at a young age and were severely affected by the mutation. A second group disrupted the CFTR coding region by neo^r insertion (65), and the resultant mice exhibited a less severe phenotype. Subsequently, the mice were shown to contain a small amount of functional CFTR protein, presumably because of a low-efficiency splicing event that removed the neo^r block in a portion of RNA transcripts (66).

Gene targeting does not necessarily mean gene ablation. Gene targeting can be used to introduce subtle mutations into a gene of interest (284), or as part of a strategy to generate a tissue-specific or inducible knockout (see sect. III*D*). Gene targeting also provides an alternative for combating temporal and spatial ectopic expression that often plague classical transgenic experiments. For example, desired mutations or gene alterations can be introduced, or "knocked in" to the endogenous mouse gene. Because expression of the mutant gene is controlled by the same factors that regulate expression of the wild-type gene, this approach avoids the confounding positional effects associated with random transgene insertion. As such, knock-in mice are potentially powerful models of human genetic diseases, caused by mutations in one (dominant) or both (recessive) alleles (see, for example, Refs. 35, 201, 221). Unfortunately, the investment of time and reagents required to generate a knock-in (or knockout) mouse are considerably greater than those required to create a transgenic mouse.

D. Inducible and Tissue-Specific Knockouts

As with transgenic studies, functional compensation could occur in a knockout mouse to mask or distort the phenotype resulting from the chronic absence of an endogenous gene. This is of particular concern when targeting a member of a large family of related genes and has been observed, for example, in mice lacking the transcription factor CREB. Knockout of CREBα and CREBδ is not lethal to mice, due in part to increased levels of CREBβ and the CREB-related protein CREM in the knockouts (19, 118). Alterations in development can occur not only at the molecular level through changes in gene expression, but also at the level of neuroanatomy. For example, lack of the dopamine D_1 receptor subtype leads to altered morphology of the striatum, in which the striosomes seen in wild-type mice are absent (293). Chronic disruption of many gene products can also result in premature death (45, 56). All three scenarios make it difficult or impossible to determine the role of a protein of interest in an adult organism.

To overcome developmental phenotypes, spatial or temporal control of gene ablation can be achieved using the *E. coli* bacteriophage P1 enzyme Cre recombinase. Cre recombinase recognizes and binds to a 34-bp DNA sequence called a *loxP* site, which contains two 13-bp repeated sequences in opposite orientation flanking an 8-bp spacer (1, 108–110). If two *loxP* sites are present in a DNA fragment, that fragment is said to be "floxed" (flanked by *loxP* sites). Cre recombinase-mediated recombination results in the removal of the entire floxed segment, leaving one *loxP* site behind (Fig. 5). Once demonstrated to function in mammalian cells (242–244), the Cre-*loxP* system was applied to mouse genetic studies (Fig. 5; Refs. 18, 97, 209, 304). For a more detailed overview of this approach, please consult Reference 282.

Recently, this method was adapted to confine gene disruption to a particular brain region and to further restrict gene disruption to postnatal ages (278, 279). Transgenic mouse lines were generated using a portion of a forebrain-specific promoter (the promoter for CaMKII-α) to drive expression of Cre recombinase (278). Mice derived from 1 of 14 founder mice expressed Cre recombinase selectively in the CA1 subfield of the hippocampus at sufficient levels to mediate recombination. Because this promoter was active only after birth, Cre recombinase expression was regulated both temporally and spatially. These mice were then crossed with mice containing a floxed *N*-methyl-D-aspartate (NMDA) glutamate receptor gene, NMDAR1, whose product comprises part of the multimeric NMDA glutamate receptor (279). The presence of the *loxP* sites, introduced by homologous recombination, did not disrupt NMDAR1 expression; however, the bigenic mice expressed Cre recombinase at high levels in the CA1 subfield of the hippocampus and therefore exhibited postnatal disruption of the NMDAR1 gene selectively in this brain region. This approach allowed the researchers to overcome the early lethality seen in classical knockouts of the NMDAR1 gene (75, 160) and to conclude

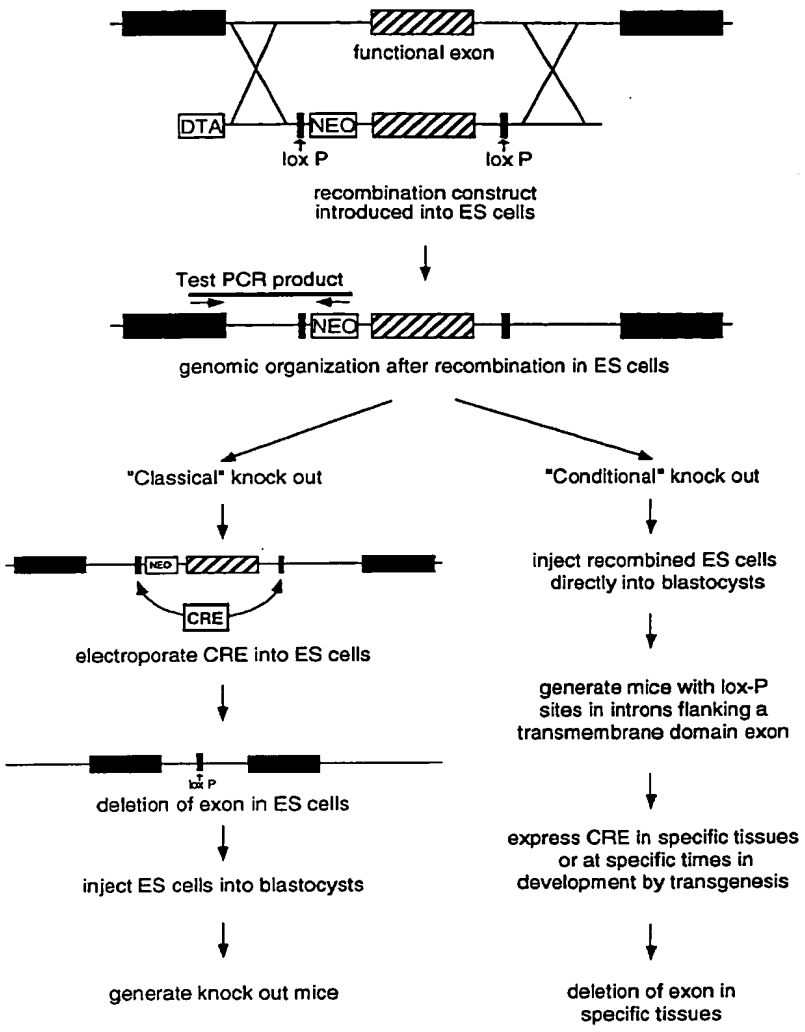

FIG. 5. Conditional/tissue-specific gene knockout. To generate a tissue-specific knockout, slight modifications in targeting construct are required. In one of several possible approaches, 2 *loxP* sites (surrounding selectable marker cassette and gene fragment of interest) are introduced. Correct recombination can be determined by PCR. ES cells can then be injected into blastocysts to generate mice with a floxed allele. When mice homozygous for floxed gene are crossed with a transgenic mouse expressing Cre recombinase in a specific tissue (brain), resultant mice will delete floxed gene only in that tissue. These ES cells can also be used to generate a "classical" knockout through transient transfection with Cre recombinase. NEO, neomycin; CRE, Cre recombinase; DTA, diphtheria toxin.

that NMDA receptor function in this subregion of the hippocampus is critical to the formation of spatial memory (183, 279).

Creating targeting constructs that function in a Cre-*loxP* system is generally no more difficult than designing a classical targeting construct. One of the distinct advantages of the Cre-*loxP* system over the classical approach, however, is the flexibility it provides the researcher. Constructs designed with the Cre-*loxP* system in mind can be used to generate mice lacking a protein in a particular tissue to avoid early lethality or severe developmental consequences, or alternatively, in a slightly modified version of the classical knockout approach (i.e., floxed alleles can be ablated in ES cells after introduction of Cre recombinase). As the technique becomes more popular, the increasing availability of transgenic mouse lines expressing Cre recombinase in various tissues and time points will allow the researcher to control gene ablation precisely. The Cre-*loxP* system has already been used to make inducible knockouts by linking Cre recombinase expression to the interferon-regulated system, further enhancing the potential for temporal regulation of gene ablation (144). In addition, Cre recombinase has been fused to a modified estrogen receptor that renders it inactive in the absence of tamoxifen, an estrogen receptor ligand (72, 73, 247). This allows temporal control over gene deletion. Although not yet reported in a neuronal system, inducible knockouts specific to brain will likely be common in the near future.

IV. MOUSE STRAIN

Many strains of inbred mice have been developed and maintained. An inbred strain is, by definition, one in which

TABLE 1. *Properties of some common mouse strains*

Strain	Sight	Hearing	Locomotion	Learning	Coat Marker	Other
C57BL/6	OK	Progressive loss	Active	OK	Nonagouti black	Used in aging models; aggressive females
129/Sv	OK	OK	Low	Poor	Agouti/brown	Defects in corpus collosum; source of most ES cell lines
DBA/2	OK	Early loss	Intermediate	Poor	Nonagouti gray	Oldest inbred strain
CBA	OK	OK	Intermediate	Poor	Agouti/brown	Used for transgenics
BALB/c	Impaired	OK	Low	Poor	Albino	
SJL	Impaired	OK	Low	Poor	Albino	Progressive myopathy develops at 6–8 mo

Learning column represents performance in Morris water maze except for the CBA strain, which was tested in radial arm maze. Note: the source of an inbred strain is critical, as isolated breeding populations alter over time (e.g., 129/SvJ performs poorly on the Morris maze, whereas 129/SvEvTac learns well). In general, F_1 crosses between any 2 of these inbred strains perform better than their parents on behavioral tests. (Compiled from References 49, 71, 151, 166, 286.)

brother-sister matings have been performed for at least 20 generations, and the line can be traced to a single ancestral breeding pair (166) (Table 1). This inbreeding results in a population of genetically homogeneous animals that are homozygous at every gene allele. The effect of mouse strain on behavior has recently received a lot of attention (86). Knockout of a particular gene can result in very different phenotypes depending on the strain used, reflecting the complex, multigenic nature of most phenotypes. In other words, if a phenotype is influenced by many genes, knockout of one relevant gene could have different effects depending on which alleles of the other genes are present. The effects can be particularly profound in behavioral paradigms (49). For example, the C57BL/6 strain is one of few inbred mouse strains that performs well in the Morris water maze, a standard test of spatial learning (286). Mice of other common strains, such as 129/Sv, perform poorly in such tests (Table 1). If a mutation in a gene of interest is studied in the context of the 129/Sv strain, and compared with wild-type mice of the C57BL/6 strain, it will seem as though the mutation attenuates learning, when in fact it is the overall genetic background rather than the specific mutation that impairs the behavior. It is, therefore, critical to use siblings of the same sex and same litter when making conclusions about the behavioral effects of a particular mutation. Even so, if genes linked (close by in the genome) to the ablated gene influence the observed phenotype, sibling pairs could show artifactual differences in behavior. In addition, many inbred mouse strains have congenital sight or hearing deficits that should be kept in mind when choosing the background strains for mutant mice (Table 1). For example, many albino strains have visual problems, and DBA/2 mice have developmental hearing impairments (49).

The profound effects of strain in a transgenic study aimed at modeling AD were recently reported. The transgene consisted of the hamster prion promoter driving expression of the amyloid precursor protein (APP) containing mutations linked to the human disorder. When C57BL/6 mice were used to generate the transgenic line, mice resulted that recapitulated many of the age-dependent hallmarks of human AD, including the appearance of amyloid-containing senile plaques with corresponding behavioral consequences (115, 190). C57BL/6 mice have been used widely for aging studies and are one of the few mouse strains that exhibit plaquelike formation (127); however, these do not contain amyloid deposits and do not correlate with neurodegeneration or deficits in learning. When the FVB strain was used to express the identical transgene, no senile plaques were observed, but the mice did exhibit a fear of novel environments (114). The differences between strains probably reflects the participation of additional genes other than APP in the formation of plaques.

Some recommendations regarding possible strategies that can be used to overcome the variability induced by strain differences have recently been proposed that may be useful in developing a breeding strategy (256). Briefly, this involves crossing mice carrying a particular mutation or transgene for several generations to mice from a defined inbred strain that shows the phenotypic characteristics relevant for the planned experiments, and using the animals only once the mutation is on a more homogeneous genetic background. If the mutation is crossed into two separate inbred backgrounds for several generations, and individuals carrying the mutation from each background are then crossed to each other, the advantages of genetic homogeneity can be achieved as well as the advantage of strong performance on behavioral tests that is usually seen in hybrids between two inbred strains. In this case, the resulting animals would be heterozygous at most genetic loci but would still be identical to each other.

V. EXISTING PARADIGMS THAT HAVE BEEN USED TO STUDY TRANSGENIC ANIMALS

Once a mutant mouse has been generated, there are any number of methods that can be used to test the neuro-

biological phenotype of the animals. It is often clear from previous biochemical or pharmacological work that complex functions might be altered in a particular mouse. In some cases however, no endogenous function has been identified for a gene product, or initial experiments have not yielded an obvious phenotype. In that case, it is of interest to consider using a battery of different physiological or behavioral tests to study the animals. An unexpected phenotype can lead to a new proposed function for a protein. In addition, many mouse models that are initially described as having no phenotype, based on results from the most obvious tests, are impaired in less expected functions. In this section we discuss some of the ways in which mouse models have been examined. This section is not designed to be comprehensive, but rather to give some examples of what has been done to analyze mutant mice, with the understanding that new paradigms are being developed constantly.

A. Physiological Measures

1. Neuronal excitability: ion channels

Channel proteins generally belong to large families containing many structurally and functionally related members (36, 67, 210, 252). Native ligand-, voltage-, and G protein-gated channels are typically multimeric complexes incorporating two or more related members of the primary channel-forming subunit family (124, 143, 210, 292). For example, the family of ionotropic (channel-forming) glutamate receptors consists of at least 14 distinct genes, whose products assemble as hetero- and possibly homopentameric complexes in vivo (262). Because neuronal subpopulations often express multiple members of the same channel family, ablating a single member of a channel family might have little or no effect at the whole animal or single-cell level. Nevertheless, the studies that have been done have enhanced our understanding of synaptic transmission and plasticity in ways that pharmacological or heterologous expression approaches could not.

Neurons can generate fast (action potentials) and slow (postsynaptic potentials) electrical signals, reflecting changes in membrane potential. Both types of signals can be monitored by intracellular recording using patch clamping or intracellular electrodes, or by extracellular recording close to a cell or cell population (106). These electrophysiological techniques are often applied to phenotypic screening in mouse genetic studies. Typically, such studies are performed on acutely dissociated neurons, cultured neurons, or on cells or cell fields within a brain slice preparation. Each preparation has unique advantages and disadvantages. Because of the restricted time frame involved in acute dissociation studies, for example, one can study the normal complement of ion channels. This can be a critical issue, since some conductances, such as G protein-gated potassium channel current activated by GABA in hippocampal pyramidal neurons, disappear in long-term culture (214, 299). Generally, patch-clamp studies on isolated single cells permit better control over intra- and extracellular conditions, better space clamp allowing for more precise measurement of current-voltage characteristics, and more rapid solution exchanges. Of course, normal interneuronal connections are lost in acutely dissociated or cultured neuron preparations. Many brain slice preparations, including the hippocampal, cerebellar, and neocortical slice, are well suited for electrophysiological studies because of their clearly defined circuitry and organization (9, 136, 184). Classically, brain slice studies have been performed in larger mammals or rats, but adaptations to these techniques for preparation and study of mouse brain slices have been made in most cases (81). Slice preparations preserve most of the normal synaptic contacts and offer some of the advantages of in vitro conditions, including adequate control over intra- and extracellular milieu. Unfortunately, rapid solution exchanges and accurate space/voltage clamp due to the extensive arborization of neuronal processes and electrical coupling between neurons can limit the utility of slice preparations for studying ion channels.

One class of ion channel that has benefited and will continue to benefit from gene-targeting approaches is the G protein-gated potassium channel (K_G). The application of knockout technology to the study of native K_G is particularly useful, since specific pharmacological probes are not available for this class of channel. The K_G are distributed throughout the CNS (132, 199, 206), where they are activated by a variety of neurotransmitters and hormones that stimulate pertussis toxin-sensitive G proteins. Accumulating evidence argues that these channels are tetrameric entities comprised of homologous subunits from the GIRK potassium channel subfamily (46, 143, 258), which consists of four members (GIRK1–4) (67). Evidence from heterologous expression systems and recent knockout studies indicates that although GIRK1 probably comprises an integral component of native K_G, GIRK1 cannot form a functional channel alone (104, 143, 287). Thus recent knockouts have targeted the other members of the GIRK family, specifically GIRK2 and GIRK4 (254, 287).

GIRK2 has received considerable attention given the identification of a point mutation in the predicted channel pore sequence that segregates with the phenotypes found in the *weaver* mouse, a model in the field of developmental neuroscience (187, 211). The GIRK2-deficient mice are susceptible to seizures but do not exhibit the profound cerebellar granule cell degeneration, disturbances in gait and coordination, or male-related infertility observed in *weaver* mice (254). This discrepancy alone indicated that the *weaver* phenotypes result from a gain-of-function mechanism related to GIRK2-containing channels. Indeed, considerable electrophysiological evidence from acutely

dissociated cerebellar granule cells as well as heterologous expression systems supports this hypothesis (140, 202, 259, 260). More recently, GIRK2 knockout mice were used to address more general questions of the role of K_G in synaptic transmission (165). Field potential and whole cell patch-clamp experiments in hippocampal slices isolated from GIRK2 knockout mice indicated that GIRK2-containing channels mediate a large fraction of the postsynaptic hyperpolarization induced in hippocampal CA1 and CA3 neurons by several neurotransmitters including baclofen (GABA), adenosine, and serotonin. In contrast, paired-pulse facilitation experiments, a postsynaptic measure of presynaptic transmitter release (171, 305), did not reveal role for GIRK2 (and K_G) in the presynaptic inhibition of neurotransmitter release by a similar panel of neurotransmitters. Given the altered electrophysiological properties identified in the hippocampus, it will be interesting to discover whether associated behavioral phenotypes related to memory or learning are observed.

In the above studies, functional compensation by the residual family members did not occur, and clear electrophysiological phenotypes were revealed. Even when functional compensation produces no phenotype or the phenotype is subtle, however, important information can be obtained from genetically altered mice that can complement antibody, pharmacological, or heterologous expression studies. For example, the evolutionary rationale for channel heterogeneity is unknown, as is the subunit composition and rules governing the assembly of most native channels. Such issues have been addressed using gene targeting of subunits of the $GABA_A$ and NMDA glutamate receptor channels in mice (Table 2). The $GABA_A$ ion channels are the principal mediators of inhibitory synaptic inhibition in the CNS. Glutamate receptor channels of the NMDA class are important for synaptic transmission and plasticity and are thought to underlie learning, memory, and development. In both cases, native channels are pentamers.

The $GABA_A$ channels assemble from the available repertoire of α-, β-, γ-, and δ-subunit isoforms. Cerebellar granule cells express six of the $GABA_A$ receptor subunits (α_1, α_6, β_2, β_3, γ_2, and δ). Although ablation of α_6 did not impair mouse motor skills, the knockout mice did exhibit a posttranscriptional loss of δ, suggesting a specific association between the α_6- and δ-subunits. Because the remaining subunits could not compensate for the lack of the δ-subunit, this observation indicated that some subunit combinations may not exist in wild-type neurons (126).

The NMDA receptors are composed of the R1 subunit and one or more of the following subunits: NR2A/ϵ_1, NR2B/ϵ_2, NR2C/ϵ_3, and NR2D/ϵ_4. The precise combination of subunits imparts unique electrophysiological properties to the complex and, thus, to the host neurons that express them. Accordingly, patch-clamp recordings from granule cells in thin cerebellar slices revealed a wide range of single-channel conductances of channels activated by NMDA in wild-type mice (70). In NR2C/ϵ_3 knockouts, the NMDA receptor-mediated whole cell current in cerebellar granule cells was only slightly affected. However, at the single-channel level, it was evident that the low-conductance variety of NMDA channels was absent. Studies like these can provide insight into the natural composition of complex channels and identify the contribution of specific subclasses of channels to macroscopic currents.

In addition, mutant mice can be used to resolve physiological controversies that have not been unraveled using traditional methodology. Mice lacking the cyclic nucleotide-gated (CNG) channel found in olfactory neurons were recently used to resolve a controversy regarding the underlying signal transduction pathway triggered by odorants (28). In in vitro biochemical assays, odorants either stimulate production of cAMP or inositol 1,4,5-trisphosphate (IP_3) through activation of G proteins. Adenosine 3′,5′-cyclic monophosphate causes membrane depolarization by stimulation of the CNG channel, whereas IP_3 was proposed to gate a different plasma membrane channel in olfactory cilia. In mice lacking the CNG channel, a panel of odorants (including some classified as IP_3 generating) did not depolarize olfactory neurons. This study suggested that activation of the CNG channel by cAMP is the primary signal transduction event involved in olfaction. A similar anosmic phenotype was observed in mice lacking the G protein α-subunit G_{olf}, identifying G_{olf} as a mediator of the cAMP production that stimulates CNG (16).

Although most genetic studies involving ion channels have involved knockout mice, transgenic approaches have also enhanced our understanding of the role of channels in neurotransmission and plasticity, generally with respect to previously characterized neurological diseases. At the adult mouse neuromuscular junction, nicotinic ACh receptors (nAChR) are pentamers composed of two α-, one β-, one γ-, and one δ-subunit (83). Transgenic mice expressing a mutant δ-subunit exhibited reduced spontaneous miniature end-plate current (MEPC) amplitudes and prolonged current decay phases (91). This and other electrophysiological evidence supported the contention that abnormal nAChR underlie the altered electrophysiological phenotype observed in the slow-channel syndrome. Channel function can be disrupted using transgenic approaches if dominant negative versions of a channel subunit exist. Dominant negative mutant proteins inhibit the function of their wild-type counterparts. Transgenic mice expressing a dominant-negative subunit of the pancreatic potassium channel that regulates insulin secretion exhibited profound deficiencies in glucose homeostasis and a deficiency in β-cells (adult mice) (185). Both resting membrane potential and basal calcium concentrations were elevated in the insulin-secreting β-cells, indicating compromised activity of the normal potassium

TABLE 2. *Glutamate receptor knockouts*

Receptor	CA1 LTP	Morris Maze Performance	Other	Reference No.
		Metabotropic		
mGluR1	Reduced	Impaired	↓ Coordination/cerebellar LTD	7, 8, 43, 44, 113, 131
mGluR2	OK	OK	Impaired mossy fiber LTD	298
mGluR4	ND	ND	↓ Coordination/cerebellar plasticity	212
mGluR6	ND	ND	Visual deficits	176
		Ionotropic		
NMDA				
NR_1	Absent	Impaired	Constitutive KO is lethal; CA1 KO has learning deficits	75, 160, 183, 276, 279, 298
NR_{2A} or ϵ_1	Decrease with age	ND	Uncoordinated when both NR_{2A} and C are KO	122, 129, 240
NR_{2B} or ϵ_2	ND	ND	Poor suckling, no CA1 LTD, no barrel formation	145
NR_{2C} or ϵ_3	ND	ND	Uncoordinated when both NR_{2A} and C are KO	129
NR_{2D} or ϵ_4	ND	ND	Reduced activity	119
AMPA/kainate				
GluR2	Enhanced	ND	Uncoordinated	125
Other				
GluRδ2	ND	ND	↓ Coordination/cerebellar LTD	133

NMDA, *N*-methyl-D-aspartate; LTD, long-term depression; KO, knockout; ND, not determined; LTP, long-term potentiation.

channel. Conditional expression of dominant negative channel subunits could be particularly useful in the CNS as a means to avoid functional compensation observed in channel subunit knockouts.

2. *Vesicle fusion and neurotransmitter release*

The use of knockout mice has clarified the function of several proteins involved in the events leading to neurotransmitter release. In recent years, molecular biological techniques have been used to identify many components of the complexes involved in synaptic vesicle trafficking, docking, fusion, and release (reviewed in Refs. 31, 96, 123). Some of the breakthroughs in this field were made by examining the components of the secretory system in yeast (reviewed in Ref. 17), because yeast are readily amenable to genetic manipulation. The obvious drawback of the yeast system is the lack of a neuronal synaptic junction. Knockouts of proteins that have been well characterized in biochemical, cell biological, and anatomic experiments in mice have allowed an examination of their role in a neuronal in vivo system. The genes that have been targeted fall into three categories: those involved in docking and fusion complexes, trafficking to the synaptic junction, or organizing the cytoarchitecture of the synaptic region.

Vesicle-associated proteins that have been knocked out include synapsins I and II, synaptotagmin I, synaptophysin, and Rab3A, all proteins thought to be involved in the regulation of neurotransmitter release or vesicle recycling (Fig. 6). From knockout experiments, it appears that, of these, only synaptotagmin I is indispensable for calcium-dependent neurotransmitter release (85). Although mice lacking synaptotagmin I die within 48 h of birth, it was still possible to examine the neuronal phenotype of this mutation because neurons from embryos or neonatal mice could be cultured. To control for developmental abnormalities that might confound a functional interpretation of the role of synaptotagmin in neurotransmitter release, the authors showed that levels of other synaptic vesicle proteins were normal and that cells in culture could form junctions and synaptic vesicles. Whole cell patch-clamp recordings from cultured hippocampal neurons revealed that pairs of pyramidal neurons from wild-type animals, in which one neuron was stimulated and depolarization was measured in the other, showed robust synaptic responses, whereas these responses were greatly attenuated in pairs of pyramidal cells from synaptotagmin knockout animals. In contrast, nonevoked miniature end-plate potentials, as well as action potentials, were normal in mutant mice. The authors concluded that synchronous, fast, calcium-dependent release is absent in these mutant mice, which together with other biochemical evidence suggests that synaptotagmin I is the low-affinity calcium sensor involved in synaptic vesicle fusion.

The synapsin family of synaptic vesicle-associated proteins has been particularly well studied (96). These peripheral membrane proteins have been thought to play a role in regulation of neurotransmitter release by cross-linking vesicles to the actin cytoskeleton in a phosphoryla-

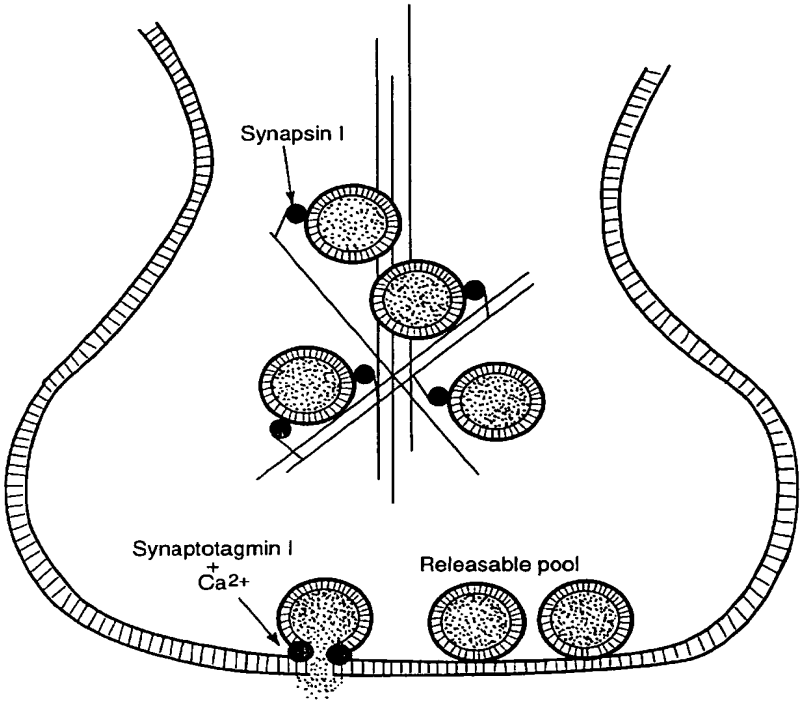

FIG. 6. Role of synapsin I and synaptotagmin I in nerve terminal. A prototypical nerve terminal is shown. Releasable and nonreleasable pools of synaptic vesicles are segregated by association of nonreleasable pool with actin cytoskeleton through synapsin I. A vesicle in process of releasing its contents into synaptic cleft in response to a Ca^{2+} signal is also shown.

tion state-dependent manner. In addition, the synapsins have been implicated in development of the synaptic terminal. Mutant mice lacking either synapsin I, synapsin II, or both genes exhibit several subtle phenotypes that rule out a direct role of the synapsins in vesicle fusion but support the idea that these proteins modulate neurotransmitter release and synaptic development as hypothesized. Electrophysiological recording from mice lacking synapsin I showed that some parameters of synaptic function were normal in the CA1 field of the hippocampus, for example, the level of stimulation necessary to evoke an excitatory postsynaptic potential. In contrast, paired-pulse facilitation, a measure of presynaptic neurotransmitter release, was enhanced at the Schaffer collateral-CA1 synapse in the hippocampus (227). These data are consistent with a role for synapsin I in segregating vesicles into releasable and nonreleasable pools, with fewer vesicles found in nonreleasable pools in the absence of synapsin I. In synapsin II mutant mice, as well as in mice lacking both synapsins I and II, further deficits in synaptic activity were seen, including decreased short-term posttetanic potentiation and synaptic depression upon repeated stimulation (228), whereas long-term potentiation (LTP), a long-lasting form of synaptic plasticity, is not affected by these mutations (265). Again, these data support the role of these proteins in segregating releasable and nonreleasable pools.

Two primary points deserve reiteration: *1*) even when a gene product is necessary for survival of the mouse, neuronal culture can yield a great deal of information from embryonic or neonatal animals; and *2*) genetic manipulation of yeast systems, which has been extremely useful in defining the pathways leading to secretion, cannot define the function of proteins specific to the neuronal synaptic junction, making mouse models invaluable.

B. Behavioral Tests

One of the great strengths of genetically altered mouse models is that one can determine how a defined protein contributes to behavior in a living animal (Table 3). Changes in behavior are rarely all or none, however, making it necessary to test large numbers of animals to obtain statistically significant results. It is also impossible to make firm conclusions from these experiments without being careful to control for differences in behavior due to background strain, developmental compensation, and age- or sex-related effects (see sect. IV). In general, behavioral tests should be carried out on same-sex pairs of wild-type and mutant littermates born to parents heterozygous for the mutation of interest. Ideally, mutant mice will be back-crossed for five or more generations to a mouse strain that has already been characterized in the behavioral test of interest, but this is very time consuming and the mutation could be lethal on the inbred background.

TABLE 3. *Common behavioral paradigms for mice*

Measure	Test	Selected Reference
Locomotion	Open field	200
	Locomotor cage	30
	Y-maze	174
	Rotorod	239
Anxiety	Light-dark box	48
	Elevated plus maze	26
	Mirrored chamber	277
Learning	Passive avoidance	208
	Radial arm maze	153
	Morris water maze	255
Drug reinforcement	Place preference	168
	Self-administration	95
	Opiate withdrawal	167

Because of these difficulties, initial characterization is usually carried out on littermates from a mixed genetic background. Because most inbred strains show deficits in performance of some complex behavioral tasks, strain selection for back-crossing can be critical.

A recent review has proposed a battery of tests that can be used to determine the neurological health of a mouse and is a useful resource for those planning to test the behavior of a new mutant mouse (50). Before behavioral testing, it is important to determine whether the animals have any sensory or motor deficits that might affect performance in complex tasks. Sensory deficits in hearing or sight can be analyzed in either very simple or more technological ways. For example, a simple test to examine hearing is to determine whether an animal startles to a loud noise like a clap. It is also possible to examine startle quantitatively using varying frequencies of sound (see, for example, Ref. 139). A simple test of vision is to observe whether an animal stops at the edge of a table or whether it simply tumbles off the edge. Performance on the visible platform test of the Morris water maze can also indicate visual acuity (see sect. v*B2*). Even if a deficit is found, it is possible to design tasks that deemphasize the deficiency. For example, one could test blind mutant mice in learning tasks that require pairing of a tone with a stimulus, rather than in a task that required visual cues.

1. Locomotion

A) LOCOMOTOR AND MOVEMENT TESTS. Locomotion is a complex behavior affected by many different brain systems, including the telencephalic dopaminergic system and the cerebellum, as well as by peripheral abnormalities (for example, muscle weakness or motoneuron degeneration). A change in locomotor activity could also result from general ill health of an animal. Because locomotor activity is required for many complex behavioral tasks, increases or decreases in locomotor activity can nonspecifically affect performance in many behavioral tests and should be measured before behavioral characterization. Although tests of locomotion are a necessary control for more complex behavioral tasks, they have also been used to study cerebellar or dopaminergic function.

Several aspects of locomotor activity can be measured including response to novelty, exploratory behavior, locomotor response to drug treatment, as well as unstimulated locomotor activity (Table 3). For example, the increased activity seen when an animal is placed in a novel environment, the number of times it rears up on its hind paws, and spontaneous alternation in a T-maze are all presumed to reflect exploratory activity (30). In contrast, activity in the home cage or after repeated exposure to an apparatus is thought to reflect overall activity more than exploration (223). A simple task that has been used to measure coordination, particularly in mutant mice suspected of having impaired cerebellar function, is the rotorod test (see, for example, Ref. 87). In this test, the animal is placed on a slim wooden rod that can rotate at variable speeds, and the latency to fall off the rod is measured. Motivation to stay on the rod can be increased by raising the rod to greater heights above a soft landing surface. This task requires intact cerebellar function and motor coordination. All measures of activity are also sensitive to the circadian clock. Mice are most active just after lights off and just before lights on (see, for example, Ref. 275), making it critical that locomotor experiments be performed at the same time of day for all subjects (for example, all experiments could be performed in the afternoon, all in the morning, or all at night just after lights off).

B) LOCOMOTOR PHENOTYPES OF EXISTING MOUSE MUTANTS. One useful function of knockout mice has been to verify the specificity of pharmacological agents with effects on behavior, or to specify more precisely the identity of the molecule mediating a particular behavioral effect of a less selective drug. The suspected target(s) of a drug can be confirmed if the drug loses its principal effect in mice lacking that target. Mouse models have shown that the locomotor-stimulating activity of cocaine and amphetamine occurs almost exclusively through inhibition of the dopamine transporter (DAT). Despite the ability of these drugs to inhibit several monoamine transporters, neither compound enhanced the activity of mice lacking DAT, as measured by photo cells in a standard rat cage (88). Similarly, mice lacking the adenosine A_{2A} receptor are hyperactive, and caffeine actually depressed locomotor activity slightly in these animals, confirming that caffeine exerts its locomotor stimulating effects by inhibiting the A_{2A} receptor (155). In contrast, the nonspecific adrenergic agonist dexmedetomidine increased locomotion similarly in mice either lacking or overexpressing the α_{2c}-adrenergic receptor, indicating that this subtype does not mediate the locomotor stimulating effects of adrenergic agonists (241). Thus genetically altered mice can be used as advanced pharmacological tools to reevaluate substances used previously in behavioral pharmacology experiments.

The combination of physiological recording from particular brain regions coupled with behavioral testing has allowed the dissection of pathways underlying motor behavior, from single molecule to physiological effect to behavioral consequences. The critical role of the dopaminergic system in controlling locomotor activity has been confirmed in mice lacking dopamine synthesis enzymes, receptors, and transporters. The ability of dopamine to control locomotor activity was first addressed by knocking out the TH gene, the rate-limiting enzyme in catecholamine synthesis (138, 302). Unfortunately, these mice die at birth because of defects in norepinephrine signaling. If the noradrenergic system is reconstituted by transgenic expression of TH under the control of the dopamine-β-hydroxylase promoter, the animals live, display normal norepinephrine synthesis, but make no dopamine (301). Mice lacking dopamine are severely hypoactive, supporting the hypothesis that activation of the dopaminergic system influences locomotor activity. In addition, disruption of the dopamine transporter, a critical enzyme in termination of dopamine neurotransmission, results in hyperactivity due to a greatly increased length of dopamine signaling, despite downregulation of many components of the dopaminergic signaling system (88).

All the knockout mice developed thus far that lack individual dopamine receptors have altered locomotor activity. Animals lacking the D_1 (293) or D_3 (2) receptors are hyperactive when tested in a standard rat cage with photoreceptors. In contrast, D_2 (13) and D_4 (236) receptor knockouts are hypoactive. A D_5 knockout has not yet been published. Although the locomotor phenotypes are largely consistent with the proposed roles of the D_2, D_3, and D_4 subtypes, these data are inconsistent with the locomotor stimulating effects of D_1 receptor agonists. It is possible that the knockout of the D_1 receptor results in compensatory changes or that it has unmasked a hitherto unknown inhibitory effect of a subsystem of D_1 receptors that is fully activated in the wild-type animal, and therefore not seen when D_1 agonists are administered. This issue remains to be resolved, perhaps by development of conditional postnatal D_1 receptor knockout mice.

Several knockout mice exhibit altered cerebellar development or impaired long-term depression (LTD) in the cerebellum, together with associated motor deficits. Long-term depression is a use-dependent decrease in synaptic strength after low-frequency stimulation that has been seen in many brain areas and is particularly well studied at the parallel fiber/Purkinje cell synapse in the cerebellum (169). For example, lack of mGluR1 (Table 2), one member of the family of metabotropic glutamate receptors highly expressed in cerebellar Purkinje cells, results in the absence of LTD in cerebellar slice recordings (8, 44). These mice also have an alteration in cerebellar development. Adult Purkinje cells are normally innervated by only one climbing fiber, but approximately one-third of Purkinje cells from mGluR1 mutant mice are innervated by multiple climbing fibers (130). The consequence of these alterations in cerebellar function and/or development is that mGluR1 mutant mice are extremely ataxic and have an abnormal gait (8, 44). In addition, mutant mice fall off a nonrotating rod very quickly and are completely unable to remain on a slowly rotating or inclined rod, suggesting poor motor coordination (8). The mGluR1 mutant mice are also impaired in the eyeblink conditioning task (8). In this motor learning task, a tone or some other stimulus is paired with a puff of air in the eye. The animal then learns to blink when the tone is presented alone. A similar phenotype (impaired motor coordination, multiple climbing fiber innervation of Purkinje cells, and impaired cerebellar LTD) has been seen upon knockout of GluRδ2, an ionotropic glutamate receptor subtype (133). In contrast, normal LTD and a milder motor impairment was seen in the mGluR4 knockout animals (212). This was coupled with an impairment of paired-pulse facilitation and posttetanic potentiation, types of plasticity involving the presynaptic terminal. These experiments demonstrate the importance of various subtypes of glutamate receptors in distinct, but perhaps overlapping, aspects of synaptic plasticity that result in a coordinated motor output.

Although less impaired in motor coordination than mGluR1 mutant mice, mice lacking the PKC-γ isoform, which is likely to be downstream of glutamate receptor activation, also perform poorly in the rotorod test (38). This deficit is associated with multiple climbing fiber innervation of Purkinje cells but not with decreased LTD (130). In contrast, mice lacking GFAP have single climbing fiber innervation of the Purkinje cells and normal coordination, coupled with impaired cerebellar LTD and eyeblink conditioning (182). It is therefore likely that some of the motor deficits seen in the various glutamate receptor mutant mice described above are a result of the multiple innervation of the Purkinje neurons and that impaired LTD is primarily responsible for the abnormal motor learning in the eye-blink paradigm.

2. Learning and memory

A) BEHAVIORAL MEASURES OF LEARNING IN THE MOUSE. Learning is a complex phenomenon subserved by the activity of many brain regions. Some aspects of learning that can be measured in rodents include attention, working memory (the short-term memory used while a task is being performed), memory consolidation, and long-term memory (lasting from 24 h to the lifetime of the animal). Various tests have been developed that evaluate preferentially one or another of these aspects of learning (Table 3). For example, the radial arm maze, in which rodents are trained to visit a pattern of arms in the maze to receive a food reward, is particularly geared toward measuring working memory (159). The animal must keep in mind

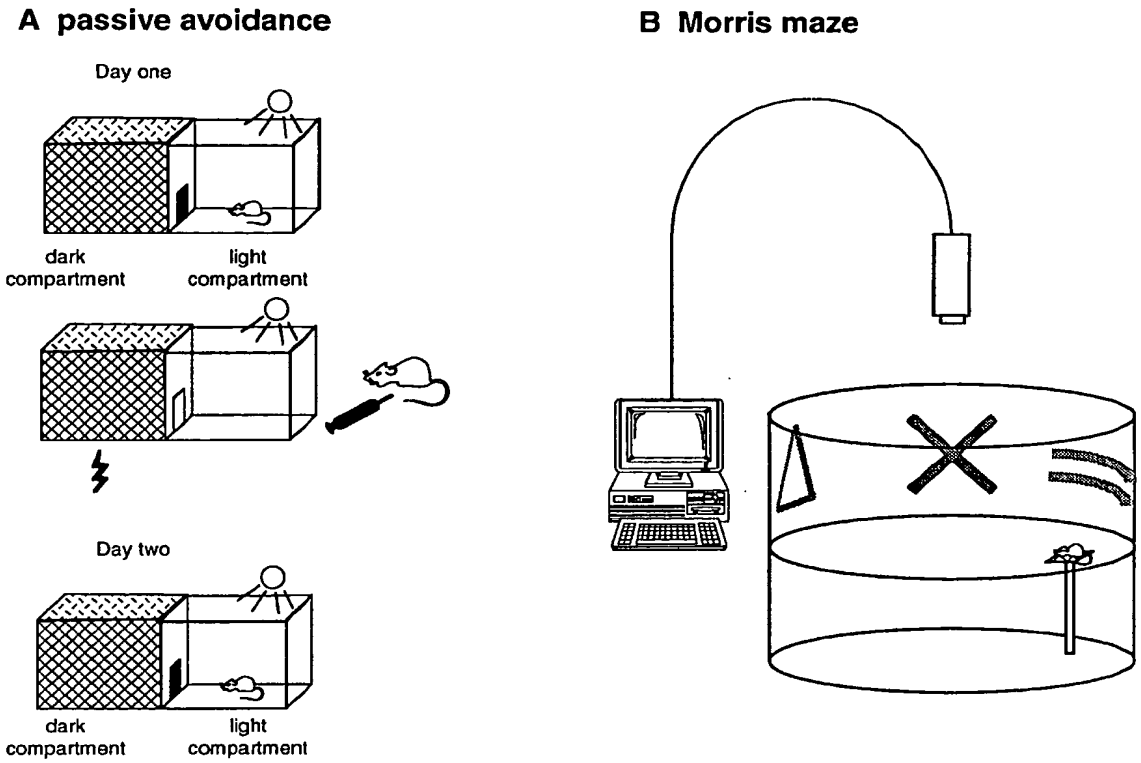

FIG. 7. Common learning tests. *A*: passive avoidance apparatus and procedure for testing associative aversive memory. On *day 1*, animal is allowed to explore apparatus. Once it enters dark chamber, a foot shock is administered. If memory consolidation is to be measured, a drug can be administered directly after foot shock. Twenty-four hours later, latency to reenter dark chamber is measured. *B*: Morris water maze. Animal is placed in water and learns to use 3-dimensional cues to find a platform hidden beneath surface of water. After several training trials, animal learns to find platform. Memory can then be tested by removing platform and measuring amount of time animal spends exploring quadrant that used to contain platform, or number of times animal crosses site of platform. Marking platform with a flag in visible platform test can control for physical abnormalities and deficits in nonspatial learning.

which arms of the maze it has already visited during the course of the task to perform well. The Morris water maze, in which an animal uses three-dimensional cues in the testing room to learn to find a hidden platform in a swimming pool, measures spatial learning which is critically dependent on hippocampal function (245). The test involves repeated trials in which the animal is placed in different parts of the pool, and the time taken to find the hidden platform is measured (Fig. 7). Plotting the time to find the platform on successive trials generates a learning curve that can be used to compare the acquisition of the spatial learning task between animals. Long-term memory can also be measured 24 h or more after the final training trial in a task called the transfer test. In this task, the hidden platform is removed, and the time that the animal swims in the area where the platform used to be is measured.

Aversive learning can be measured using the passive avoidance task, and memory consolidation can be examined by administering drugs immediately after training (208). This one-trial test of aversive memory pairs a mild foot shock with entry into a dark chamber (Fig. 7). Twenty-four hours after the foot shock, the length of time the animal avoids the dark chamber is measured to determine memory of the foot shock. Drugs such as nicotine administered directly after the foot shock will enhance the avoidance behavior (208). This task depends on fear of the foot shock and involves activation of the amygdala (224). Lesion studies and dopaminergic antagonists have also shown that both passive avoidance (68, 268) and active avoidance, in which the mouse is trained to avoid a shock (116), are dependent on intact striatal function. As an example of the use of these types of learning tests, mice lacking the G protein exchange factor Ras-GRF show impaired performance 24 h after passive avoidance training, that is, they did not avoid the dark chamber as long as wild-type mice (24). In contrast, mice without the β_2-subunit of the nAChR show enhanced retention of avoid-

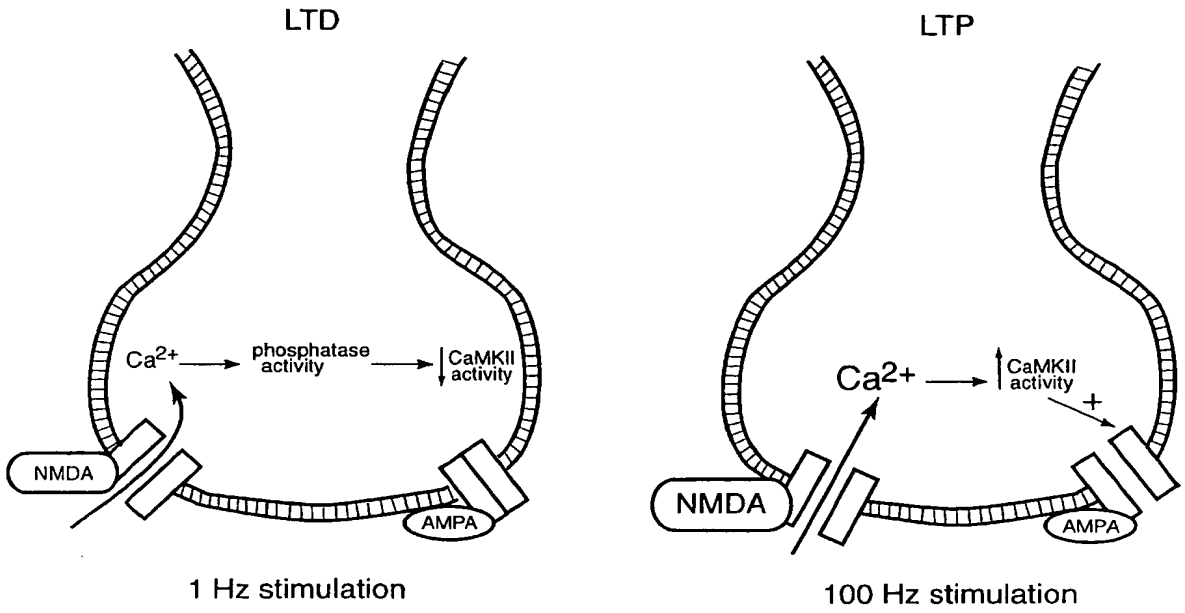

FIG. 8. Establishment of long-term depression (LTD) and long-term potentiation (LTP). In 1 model, low-frequency stimulation (1 Hz) of a synapse leads to a small rise in intracellular Ca^{2+} through *N*-methyl-D-aspartate (NMDA)-type glutamate receptors. This rise in Ca^{2+} is too low to stimulate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activity significantly and does not result in long-term activation of enzyme. Instead, activation of Ca^{2+}-dependent phosphatase calcineurin occurs, inducing a phosphatase cascade. These processes lead to LTD. In contrast, high-frequency stimulation (100 Hz) allows more Ca^{2+} to enter cell through NMDA-type glutamate receptors. CaMKII is activated leading to long-term activation of enzyme by autophosphorylation. CaMKII can phosphorylate and increase probability that DL-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA)-type glutamate receptors will open, further depolarizing neuron. These processes lead to LTP. [Adapted from Malenka (169).]

ance 24 h after training and show no response to nicotine which potentiates the avoidance behavior in wild-type animals (215). Contextual learning can also be measured in a manner that minimizes the role of locomotor activity in the behavior. For example, contextual freezing, in which the animal is administered a foot shock in a distinctive chamber and then examined for fear-induced freezing in the same environment 24 h later, is a hippocampal-dependent task that works well even in animals that have impairments in locomotor activity (39). Many such tests can be found in the literature, and most have been tried at least once with mice (Table 3).

B) PHENOTYPES INVOLVING LTD, LTP, AND SPATIAL LEARNING. One of the most fruitful areas of research using transgenic and knockout mice has involved the biochemical and cell biological basis of spatial learning. Considerable work has focused on the link between hippocampal LTP and performance on spatial learning tasks (reviewed in Refs. 40, 179). A hallmark of hippocampal electrophysiology is LTP (Fig. 8), the phenomenon whereby brief repetitive (tetanic) stimulation of an afferent pathway or presynaptic neuron, concomitant with postsynaptic depolarization, induces a long-lasting increase in synaptic strength (20, 204). Long-term depression is a related use-dependent phenomenon characterized by a persistent, decreased synaptic strength (169) (Fig. 8). The most common form of LTP, typically measured at the Schaffer collateral/CA1 pyramidal neuron synapse, requires NMDA receptor function to increase postsynaptic calcium levels and subsequent activation of phosphorylation cascades.

Many receptors (particularly ionotropic glutamate receptor subtypes), signaling molecules, and growth factors suspected of playing a role in neuronal plasticity have been knocked out in mice resulting in disruption of LTP, LTD, spatial learning, or all three. As mentioned above, several genes, including mGluR1 (Table 2) and GFAP, influence LTD in the cerebellum as well as forms of motor learning. Both of these knockouts also affect LTP in the CA1 region of the hippocampus (7, 182). Calcium/calmodulin-dependent protein kinase II is activated by entry of calcium through NMDA receptors after stimulation that leads to LTP (for review, see Ref. 169) (Fig. 8). The enzyme remains active for many hours in the absence of calcium as a result of autophosphorylation, suggesting that this enzyme might act as a switch inducing LTP. The knockout of CaMKII provided the first example of impaired LTP coupled with a deficit in spatial learning using the Morris water maze (255, 257). This story was slightly

complicated by the subsequent report that CaMKII mutant mice were highly susceptible to limbic epilepsy, which could have contributed to the deficits seen (29). Subsequently, a constitutively active form of CaMKII was overexpressed in the forebrain by transgenesis. This artificial elevation of CaMKII activity resulted in animals that showed normal LTP at 100-Hz stimulation, but at lower stimulus frequencies (1, 5, or 10 Hz), transgenic animals showed a lack of LTP and a shift toward more pronounced LTD than was seen in wild-type animals (181). The shift in the threshold between LTD and LTP was accompanied by a deficit in spatial memory (12). These mice also showed abnormal formation of place fields, areas defined by the firing of hippocampal neurons called place cells when the mouse is in a particular area of the maze (230). This result was confirmed using animals that expressed the CaMKII transgene under the control of an inducible, Tet-sensitive promoter (180). Normal LTP and spatial learning was restored by turning off expression of the transgene, implying that the expression itself, rather than adaptation to the transgene, was responsible for the phenotype.

A similar experiment was performed by expressing a truncated form of calcineurin, a calcium-dependent phosphatase, primarily in the hippocampus (173, 288). Whereas activation of CaMKII (which would increase phosphorylation of its targets) is thought to be necessary for induction of LTP, activation of calcineurin by low levels of calcium (which would decrease phosphorylation of its targets) is thought to be necessary for setting the balance between LTD and LTP (169) (Fig. 8). Overexpression of calcineurin resulted in a decrease in CA1 LTP induced by multiple trains of 100-Hz stimulation (288) and a concomitant impairment in long-term memory (173), supporting the hypothesis that this molecule is involved in LTP, but leaving open the question of whether it can act as a switch between LTP and LTD.

The NMDA receptor, which is made up of NR1 and at least one NR2-type subunit (A-D), has long been considered to be central to many types of LTP (15). The postnatal, CA1-specific knockout of the NR1 subunit has been very informative. Total knockout of NR1, a necessary component of all NMDA receptor subtypes, results in perinatal death of the animal (160). In contrast, knockout of this subunit postnatally only in the CA1 subfield of the hippocampus (as described in section III*D*) results in abolished LTP in CA1 and impaired spatial memory (279), as well as unstable place fields, as measured with an array of extracellular electrodes measuring place cell activity in the hippocampus (183). The NR_{2A} subunit appears to be its partner in the hippocampus, because knockout of this subunit also resulted in reduced CA1 LTP and impaired spatial learning (240). The non-NMDA glutamate receptors also play a role in LTP and learning. Intriguingly, knockout of the GluR2 subunit, which generally reduces the calcium permeability of DL-α-amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA)-type receptors, results in enhanced LTP (125). Unfortunately for those looking to make a smarter mouse, these animals die earlier than wild-type mice and have impaired motor coordination and exploration.

Many knockout mice have now been tested for their performance in learning paradigms. Although some of the outcomes of these experiments were expected (for example, that NMDA receptors and CaMKII play a role in LTP and learning), these experiments are helpful in proving hypotheses about learning. These types of experiments have also shown that although LTP in the CA1 region of the hippocampus is correlated with intact spatial learning (279), LTP in the CA3 region or the mossy fibers is not absolutely required for spatial learning (see, for example, Ref. 207). Ultimately, these experiments will identify how molecules involved in learning pathways work together to cause activity-dependent plastic changes.

3. Reinforcement

A) REINFORCEMENT PARADIGMS. Drug abuse is one of the human health problems best replicated in animal models as several paradigms exist for measuring the addictive properties of drugs in rodents. These range from measuring the rewarding properties of drugs (178) to measuring withdrawal symptoms (167) after chronic treatment with drugs of abuse (Table 3). The place preference paradigm can be used to test both the reinforcing properties of drugs as well as the aversive symptoms of withdrawal. In this test, injection of a drug is paired with placement of the mouse in a particular environment in a multi-compartment apparatus, whereas saline injection is paired with a second compartment. Repeated pairing of a drug of abuse like cocaine or morphine with one compartment will cause an animal to explore that environment for a longer time when allowed to choose freely between compartments, reflecting the reinforcing properties of the addictive drug (see, for example, Ref. 168). In contrast, withdrawal can be induced and paired with exposure to a particular environment, and avoidance of the withdrawal-paired compartment can be observed. This paradigm involves spatial learning and locomotion, so if either of these processes are impaired, changes in place preference may occur. This test can be performed using either an unbiased design, in which both compartments are equally preferred by the animal, or a biased design, in which one compartment is preferred at the beginning of training. In a biased design, the outcome is affected by the anxiety state of the mouse; drug administration may relieve the anxiety associated with the less-preferred compartment resulting in enhanced place preference.

Perhaps the best test of drug reinforcement is the self-administration paradigm that measures how hard an

animal will work to obtain a drug of abuse. In this test, the mouse must press a lever or poke its nose into a hole, one or a number of times to receive the drug (in mice, see, for example, Refs. 62, 216). This paradigm is particularly useful as it can show for an individual animal the acquisition of drug-seeking behavior, the extinction of that behavior once the drug is withdrawn, and how hard the animal is willing to work to receive the drug. Again, the animal must learn how to perform the task, so deficits in learning must be controlled for, perhaps by teaching the animal how to self-administer food after food-deprivation.

B) EFFECTS OF MOUSE MUTATIONS ON DRUG REINFORCEMENT PATHWAYS. Recently, several molecules suspected to play a role in the reinforcing properties of drugs of abuse have been knocked out. These molecules include transporters and receptors involved in dopamine neurotransmission, receptors for various addictive substances such as opioids, psychostimulants, or nicotine, and other signaling molecules that transduce or modulate messages in reinforcement pathways. The current hypothesis is that drugs of abuse exert their reinforcing properties through a common pathway involving an increase in the activity of dopaminergic neurons and the amount of extracellular dopamine in the nucleus accumbens (141). Different drugs of abuse achieve this end point through interactions with distinct target molecules, however. Knockout mice have been generated lacking molecules that affect shared reinforcement pathways as well as molecules that mediate the effects of particular drugs of abuse.

Some of the interesting knockout mice generated to study common pathways activated by drugs of abuse are in the dopaminergic system. One of the more surprising results seen is that mice lacking the dopamine D_1 receptor were no longer affected by psychomotor stimulants on tests of locomotor activity (191) but still showed normal place preference for cocaine (186). Controls for these experiments included demonstration that the D_1 knockout mice, which are hyperactive, could increase their activity at night, but that daytime activity was unchanged in response to cocaine or amphetamine (191). The D_2 receptor mutant mice, in contrast, are still responsive to the locomotor stimulating properties of psychostimulants (13), but not to the rewarding effects of cocaine (168). As a control, food reward could still condition a place preference in these animals. This indicates that the two primary functions of psychomotor stimulants, stimulation of locomotor activity and drug reinforcement, can be disassociated by ablation of specific genes.

Mice have also been developed that lack molecules necessary for the response to specific categories of drugs of abuse. For example, mice lacking the μ-opioid receptor are insensitive to the rewarding effects of morphine (178). These mice are also insensitive to the analgesic properties of opiates (178, 263) as well as the locomotor-stimulating response of morphine (274), implying that the initial event in all these responses to opiates is activation of the μ-opioid receptor. In addition, nicotine reinforcement has been studied in mice lacking 1 of the 11 known subunits of the nAChR expressed in neurons (215). Nicotine-induced dopamine release as well as an electrophysiological response to nicotine in dopaminergic neurons was absent in β_2-mutant mice, implying that this effect is dependent on a receptor containing the β_2-subunit (216). In addition, β_2-mutant mice self-administer cocaine, but not nicotine. Like the experiments with the μ-opioid receptor knockouts and morphine reinforcement, these experiments identified a necessary component of the receptor that initiates the pathway to reinforcement. In contrast, the dopamine receptor knockouts might be expected to be deficient in reinforcement for many categories of drugs of abuse.

C. Mouse Models of Human Neurological and Psychiatric Disease

There are several advantages to a mouse model of human disease, including genetic homogeneity and environmental uniformity, that can eliminate variables facing a large-scale human study. Disease onset and progression can therefore be studied in a highly controlled environment. Early stages of the disease, before the appearance of overt symptoms, are also amenable to examination. A good mouse model of a human disease exhibits a measurable phenotype relevant to the human pathology. For example, neurologically based movement disorders can be modeled by looking for the pathology in the brains of the animal model and then measuring locomotion in the impaired animals. For many psychiatric diseases, which may have several causes, it is not possible to have one model that mimics all the aspects of the human disease. Accordingly, it may not be possible to know whether one has a schizophrenic mouse. Instead, it might be possible to break down the human disease into cognitive and attentional deficits, negative symptoms, and psychosis, and perhaps individual mouse mutants can be developed that model particular subsets of symptoms of the disease.

A number of behavioral models have been developed to mimic human psychiatric illness such as depression and some aspects of schizophrenia. One aspect of schizophrenia that can be reproduced in an animal model is loss of auditory gating (266). Two tones presented very close together will not evoke a response to the second tone in most people, but schizophrenic patients will respond to both tones (3). In rodents, a very soft tone that will not startle the animal can be presented just before a loud tone and will significantly depress the startle response to the loud tone (267). In knockout studies, prepulse inhibition of startle has been shown to be slightly increased in mice lacking the serotonin$_{1B}$ receptor, im-

plying that this receptor subtype modifies prepulse inhibition in wild-type mice (69).

Depression models primarily depend on unavoidable stress to induce a depressed state in the animal. For example, in the learned helplessness paradigm, which has been performed using mice, an animal repeatedly exposed to an inescapable foot shock will not try to escape when given the opportunity (10, 281). Antidepressant treatment can reverse this effect. Although these depression models have not yet been applied to transgenic or knockout animals, many mouse lines exist that will be examined in these models in the future.

Despite the progress in developing behavioral paradigms to probe psychiatric disease, genetically altered mice can be used to model human genetic diseases in a more straightforward fashion (5, 152, 156). Genetic disorders are caused by chromosomal mutations that affect the expression level or function of a particular gene(s). Many factors influence the onset and progression of most diseases. Multiple lines of mutant mice may be required, therefore, to mimic all facets of a multifactorial disease. Two neurological diseases that have been studied extensively with transgenic and knockout mice are ALS and AD. Here we briefly review the progress toward modeling these diseases in mice, highlighting the key contributions of mouse studies toward understanding the underlying disease mechanisms.

1. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis is an adult-onset disease characterized by degeneration of motoneurons in the cortex, brain stem, and anterior horn of the spinal cord (14, 58). After diagnosis, usually in the fourth or fifth decade, ALS proceeds toward paralysis and death due to respiratory failure. There are no early markers for ALS, making attempts to reveal causative factors in the human disease virtually impossible. Approximately 5–10% of all ALS cases are inherited in an autosomal dominant fashion (familial or fALS). The large majority of ALS cases, however, are sporadic in nature. Several hypotheses (discussed below) have been forwarded as explanations for the delayed onset and progression of ALS, as well as the specific ablation of motoneurons, and transgenic mouse models have proven invaluable in the direct testing and refinement of these hypotheses (27, 99, 117, 164).

A link between 20% of fALS cases and oxygen free radical scavenging was established with the identification of mutations in the copper/zinc superoxide dismutase (SOD1) gene in ALS-afflicted family members (60, 229). Superoxide dismutase 1 is a ubiquitously expressed cytosolic member of a family of enzymes that catalyzes the conversion (dismutation) of superoxide to hydrogen peroxide (77). Over 50 different fALS-associated mutations in SOD1 have been identified (60, 229, 289). Transgenic and knockout studies revealed that SOD1-related ALS results from a gain of function associated with the mutant SOD1 protein, rather than a loss of ability to convert oxygen free radicals. Mice lacking SOD1 exhibited a subtle phenotype; although motoneurons were susceptible to oxygen free radical damage, there was no degeneration of motoneurons in vivo (220). On the other hand, transgenic mice expressing several fALS-associated human SOD1 mutations, but not wild-type SOD1, displayed progressive muscle atrophy associated with motoneuron degeneration leading to paralysis and death (41, 54, 101, 225, 280, 290). Importantly, virtually unaltered levels of SOD1 activity were observed in these mice, indicating that the SOD1 mutations did not exert a dominant negative influence on endogenous murine SOD1. In these studies, the transgenes consisted of the complete SOD1 gene, including its own promoter, enhancer, and introns. Nevertheless, the observed syndromes typically differed with respect to time course and severity, likely reflecting different transgene copy number or integration site. For example, mice expressing one mutant form of SOD1 (G93A) show disease symptoms between 90 and 135 days of age and died before 190 days of age and presented histopathological markers that were at variance with human ALS (41). In contrast, mice containing a lower copy number of the same transgene and expressing less of the mutant SOD1 protein survived more than 400 days and produced histological changes virtually identical to those observed in human patients (54).

Mutations in SOD1 alone cannot account entirely for the specific ablation of motoneurons observed in ALS, because SOD1 is expressed ubiquitously. Thus many studies have been directed toward identification of motoneuron susceptibility factors. The abnormal accumulation of neurofilaments is an early histopathological observation in ALS patients (reviewed in Ref. 107). Neurofilaments are the most abundant cytoskeletal structures in large myelinated axons arising from large motor and sensory neurons and are composed of three intermediate filament proteins ranging between 60 and 120 kDa: NF-L, NF-M, and NF-H. Neurofilaments maintain axonal integrity in cells that possess long processes such as motoneurons, whose axons may extend for over a meter. A possible link between neurofilament proteins and the pathogenesis of ALS was supported by studies of transgenic mice overexpressing murine NF-L (294) or wild-type human NF-H (47), both of which resulted in the accumulation of neurofilaments in motoneuron cell bodies and denervation of skeletal muscle. However, neither model exhibited significant loss of motoneurons. Expression of an assembly-disrupting mutant version of NF-L, however, led to selective degeneration of spinal motoneurons accompanied by the accumulation of neurofilaments and denervation of skeletal muscle (157). This demonstrated that neurofilament mutations can give rise to specific degeneration of moto-

neurons and muscle wasting. A more direct causative link between ALS and neurofilament dysfunction was suggested when variant neurofilament (NF-H) alleles were identified in a small but significant number of sporadic ALS cases (74, 128).

Although genetic linkage studies indicate that ALS is a multifactorial disease, the clinical and histological similarities argue that most or all causes funnel through a common destructive pathway. Indeed, considerable evidence supports the contention that disturbances in glutamate metabolism represent the common destructive pathway in many or all ALS cases (231, 250, 251). One theory is that the glutamate transporter, GLT-1, responsible for clearance of glutamate, and thus the regulation of glutamate signaling, is specifically targeted in ALS patients, rendering their motoneurons susceptible to excitotoxic damage (232–235). Intriguing interactions between fALS-associated mutant SOD1 proteins, neurofilaments, and glutamate-mediated toxicity have been reported. The drug riluzole, which interferes with pre- and postsynaptic glutamate neurotransmission and has been shown to be moderately effective in prolonging survival in human clinical trials (146), was also shown to prolong the survival of a mouse expressing a fALS-associated SOD1 mutation (100). In addition, in transgenic mice expressing another fALS-linked SOD1 mutant, NF-L was selectively nitrated, disrupting the assembly of unmodified subunits (51). Thus mouse models have provided a useful starting point for examining the pathological progression of ALS and are currently being used to reveal interactions between the seemingly disparate proteins linked to the disease. Future studies using transgenic or knockout mice should elucidate the role of glutamatergic dysfunction in the etiology of ALS.

2. Alzheimer's disease

Whereas ALS is a neurodegenerative disease that primarily affects motor function, AD is a slow, multigenic neurodegenerative disease characterized by progressive deterioration of memory, cognition, language, and visuospatial skills (248). The histopathological hallmarks of this disease include extracellular senile plaques, intracellular neurofibrillary tangles, and neuronal loss. A comprehensive mouse model for AD would exhibit the histological hallmarks of AD as well as the associated behavioral deficits (162). As with ALS, both familial (fAD) and sporadic forms of AD are encountered. To date, attention has focused on a handful of potential disease-related proteins, including APP, the microtubule-associated protein tau (172), transforming growth factor-β1 (291), the calcium-binding protein S100β (222, 297), interleukin-6 (80), and the presenilins (PS1 and PS2; Ref. 102).

Senile plaques found in the brains of AD patients are extracellular deposits of heterogeneous substances including a 4-kDa peptide termed β-amyloid (Aβ or Aβ4), which is a proteolytic fragment of the APP (197). Several mutations in the APP gene cosegregate with fAD (reviewed in Ref. 195). Although little is known regarding its normal function, mice with targeted mutations in APP are smaller (15–20% lighter) than wild-type siblings because of decreased food and water intake and exhibit decreased locomotor activity and forelimb grip strength (198, 300). These studies suggest that APP is dispensable for development or that another protein can compensate for its absence. Indeed, the deficiency in APP could be compensated by the amyloid precursor-like proteins (APLP1 and APLP2), both which lack the Aβ region.

The majority of APP is processed in a nonamyloidigenic fashion (reviewed in Ref. 248). In one pathway, APP is cleaved by an unidentified protease (α-secretase) within the Aβ region, precluding formation of β-amyloid. In the amyloidigenic pathway, APP is cleaved by two unidentified proteases (β- and γ-secretases) to generate the Aβ peptide (90, 270). Familial AD-linked APP mutations increase the level, length, or fibrillogenic properties of Aβ (149). Mice expressing the Aβ peptide under the control of neurofilament light chain promoter exhibited histological defects in highly restricted brain regions that contrasted with the widespread expression of the Aβ transgene (147, 148). Thus Aβ itself may not be neurotoxic but could work in concert with other proteins (see also Ref. 194).

Although several early attempts to model AD by transgenic expression of APP mutations were unsuccessful (53, 150, 162, 170, 221), mice expressing one fAD-associated mutation in APP driven by the platelet-derived growth factor promoter (84) exhibited elevated APP mRNA and protein levels together with progressive age-related amyloid deposition (175, 226), but no neuronal loss (121). More recently, mice have been generated that develop plaques and show behavioral consequences linked to mutant APP expression, establishing a connection between abnormal APP expression and cognitive impairment (115, 190). In these mice, levels of the highly fibrillogenic Aβ peptide (Aβ42) were increased by 14-fold over endogenous levels, and learning and memory deficits preceded the histological evidence of the syndrome. No overt neuronal loss was observed in this model, however (120). These findings provide further evidence that Aβ is not acutely toxic or solely responsible for the behavioral deficits seen in AD.

Although several mutations in the APP gene are found in both sporadic and familial cases of AD, mutations in the presenilin genes (PS1 and PS2) account for a larger percentage of fAD (reviewed in Ref. 102). The PS1 mutations are linked to the most aggressive form of early-onset fAD, which produces clinical signs by age 35. Approximately 30% of early-onset fAD segregate with PS1 mutations. Presenilin 1 and PS2 are homologous integral membrane proteins containing multiple putative

membrane-spanning domains (64). Both genes are expressed in brain and various peripheral tissues, and both localize to the endoplasmic reticulum and early Golgi (142, 285). The fAD-linked mutations are found in the membrane-spanning domains and are predominantly missense in nature (102).

All fAD-linked PS1 mutations examined thus far increase the levels of Aβ42 (102, 149, 246). Transgenic mice coexpressing wild-type human APP and various fAD-linked PS1 mutants under the control of the hamster prion promoter exhibited significant overproduction of Aβ42 in the brain at 2–4 mo of age, before the appearance neuropathological evidence of neurodegeneration (42). In contrast, the expression of wild-type PS1 did not alter Aβ production. Recent studies suggest a functional link between PS1 and the γ-secretase required for Aβ generation (63). Although PS1 knockout embryos die and exhibit skeletal abnormalities (102, 249), hippocampal neurons can be cultured from PS1 knockout embryos and produce significantly less Aβ because of a specific reduction in γ-secretase activity (63). Thus mouse models have begun to identify the mechanisms underlying the pathology associated with AD. Future experiments should elucidate the relationship between the proteins linked to AD and what types of intervention can reverse or impede the neurodegeneration observed in this disorder.

VI. CONCLUDING REMARKS

Despite the rapid growth in the number of papers published using knockout or transgenic mice to study the function of the nervous system, the field is still in its infancy. These techniques are still evolving, and rapid advances in biotechnology have now made it possible to control the location of overexpression or knockout of a gene of interest, the amount of expression from a transgene, and the timing of expression or knockout. Ultimately, it would be ideal to be able to up- or downregulate the expression of a specific gene in any cell type, much like a dimmer switch regulates a light. Although this is still science fiction, it makes sense to take advantage of the flexibility of existing methods when generating new mutations. For example, the Cre-*loxP* system allows generation of either a "classical" knockout or a variety of tissue-specific and/or temporal knockouts depending on the availability of lines of transgenic mice expressing Cre recombinase in tissues of interest. Similarly, generation of a TetOp-driven transgene allows the use of multiple tTA expressing lines of mice to achieve regulated, tissue-specific expression of the transgene. As new lines of Cre recombinase or tTA transgenic mice are generated, the possibilities will expand.

The specificity of disease models will improve in coming years as mutated genes linked to disease are knocked in to replace endogenous genes. This should more accurately reproduce the expression pattern of the disease gene and more closely mimic the human disease state. In addition, use of BAC will improve the ability to generate mouse lines with desired expression patterns of the transgene.

It is important to keep in mind that even with improved technology, one knockout or transgenic mouse will not be identical to another. Copy number and insertion site are likely to influence expression pattern even when very large promoter fragments are used for transgenesis. In addition, strain differences will be an ongoing source of variability. However, the use of different background strains to examine knockout and transgenic phenotypes could lead to the identification of interacting genes that modify the function of a gene of interest.

Finally, as we approach a critical mass of mutations in pathways necessary for neuronal function, mixing and matching of mouse lines will occur. This will allow the genetic dissection of individual signal transduction pathways and the identification of sites of interaction between pathways. As has been seen in the human genome project, the convergence of information from many laboratories is likely to result in a synergistic increase in our knowledge of nervous system function.

Thank you to Dr. Andrew Spicer and Dr. David Clapham for critical reading of the mansucript.

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of the circadian cycle in mammals. Mutational analyses of other putative clock genes will be essential for unravelling the molecular mechanisms underlying the mammalian circadian clock. These mutants will also provide useful animal models for elucidating the aetiology of and developing treatments for disorders in humans related to the sleep–wake cycle. □

Methods

Generation of *mPer2*^{Brdm1} mutant mice. We isolated a genomic clone from a mouse 129S5/SvEvBrd genomic library using a mouse *mPer2* complementary DNA probe. A targeting vector was constructed with *PGK-Neo* as the positive selection marker and *HSVtk* as the negative selection marker to delete a 2.1-kilobase (kb) fragment. We used a 6.7-kb *Bgl*II fragment as the 5′ homology region and a 4.0-kb *Kpn*I fragment as the 3′ homology region. The *HSVtk*, *PGK-Neo* and vector backbone were from pKO SelectTK, pKO SelectNeo V800 and pKO Scrambler V924 (Lexicon Genetics). Tissue culture, electroporation, mini-Southern blot analysis on embryonic stem cell colonies, generation of chimaeric and germline mice and tail DNA genotyping were done as described[27,28].

Locomotor activity monitoring and circadian phenotype analysis. Mice were housed in individual cages equipped with a running wheel in ventilated, light-tight chambers with controlled lighting. Wheel-running activity was monitored by an on-line PC using the Chronobiology Kit (Stanford Software Systems). In the LD cycle, the light was turned on at 7:00 (ZT 0) and off at 19:00 (ZT 12). The switch into constant darkness (DD) was effected by not turning on the light at the usual time. The activity records are double plotted so that each day/cycle's activity is shown both to the right and below that of the previous day/cycle. Activity is plotted in density percentile distribution (Fig. 2) or threshold (Fig. 3) format. For activity counting we used the ACTCNT program of the Chronobiology Kit. To determine the period length, an interval with a 10-day minimum during which the circadian period appeared to be stable on the activity record was analysed with a χ^2 periodogram[29] using the Stanford Chronobiology Kit. We used Fourier periodogram analysis[15] in the Chronobiology Kit to assess the strength of circadian and/or ultradian rhythmicity.

***In situ* hybridization.** Mice were killed by cervical dislocation under ambient light conditions at ZT 6 and ZT 12 and under a 15 W safety red light at ZT 18 and ZT 0/24. Specimen preparation and *in situ* hybridization with an *mPer1* and an *mPer2* probe were carried out as described[4,30]. The *mPer2* probe is outside the region deleted in the mutant. The *mPer3* probe was made from an RT–PCR product corresponding to nucleotides 480–824 (AF050182). The *Clock* probe was made from an RT–PCR product corresponding to nucleotides 1352–2080 (AF000998). Tissue was visualized by fluorescence of Hoechst dye-stained nuclei (blue in Fig. 4).

Northern blot and RT-PCR analysis. Tissues were collected and frozen in liquid nitrogen and stored at −80 °C. RNA was prepared with the RNAzol™ B RNA isolation kit (TEL-TEST). We performed northern blot analysis on total tissue RNA using denaturing formaldehyde gel. For RT–PCR analysis, first strand cDNA was generated using Moloney reverse transcriptase (BRL-GIBCO) and oligo dT-priming from total liver RNA. An aliquot of the first strand cDNA was then amplified by PCR across the deletion region with the 5′ primer CTA CCT GGT CAA GGT GCA AGA G and the 3′ primer GGT TTG AAT CTT GCC ACT GG. The RT–PCR products were then sequenced with an internal primer AGG GTA CAC TCG GGC TAT GA.

Received 31 December 1998; accepted 18 May 1999.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements. We thank S. Vaishnav, L. Qiu, Y.-C. Cheah, E. Sheppeard and S. Rivera for technical assistance; P. Hastings for comments on the manuscript; J. W. Patrick for providing space and facilities for circadian phenotype analysis; and J. Takahashi, Y. Zhao and M. Bucan for helpful discussions. This work was supported by grants from NINDS and NIDA to J. W. Patrick, from the Max-Planck Society to G. E., from NIH and the Department of Defense to C.C.L., and from NIH and the Howard Hughes Medical Institute to A.B. A.B. is an investigator with HHMI.

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Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse

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Amyloid-β peptide (Aβ) seems to have a central role in the neuropathology of Alzheimer's disease (AD)[1]. Familial forms of the disease have been linked to mutations in the amyloid precursor protein (APP) and the presenilin genes[2,3]. Disease-linked mutations in these genes result in increased production of the

42-amino-acid form of the peptide ($A\beta_{42}$)[4–8], which is the predominant form found in the amyloid plaques of Alzheimer's disease[9,10]. The PDAPP transgenic mouse, which overexpresses mutant human APP (in which the amino acid at position 717 is phenylalanine instead of the normal valine), progressively develops many of the neuropathological hallmarks of Alzheimer's disease in an age- and brain-region-dependent manner[11,12]. In the present study, transgenic animals were immunized with $A\beta_{42}$, either before the onset of AD-type neuropathologies (at 6 weeks of age) or at an older age (11 months), when amyloid-β deposition and several of the subsequent neuropathological changes were well established. We report that immunization of the young animals essentially prevented the development of β-amyloid-plaque formation, neuritic dystrophy and astrogliosis. Treatment of the older animals also markedly reduced the extent and progression of these AD-like neuropathologies. Our results raise the possibility that immunization with amyloid-β may be effective in preventing and treating Alzheimer's disease.

Our initial experiments investigated the effects of immunization against amyloid-plaque-related proteins on the development of AD-like neuropathology in young PDAPP mice, in which treatment had begun before the occurrence of significant plaque pathology (6 weeks of age). The immunogens were either synthetic human $A\beta_{42}$, the major component of β-amyloid plaques in AD and PDAPP mice, or peptides derived from the primary amino-acid sequence of serum amyloid-P component (SAP). SAP is a protein associated with amyloid plaques in AD and other amyloid diseases[13]. Two additional groups of PDAPP mice were immunized with PBS buffer or were left untreated to serve as controls. Mice received 11 immunizations over an 11-month period. We found that eight of nine PDAPP mice immunized with $A\beta_{42}$ developed and maintained serum antibody titres against $A\beta_{42}$ of greater than 1:10,000. The ninth mouse had a lower titre, of approximately 1:1,000. Cross-reactivity of the antisera was also observed against mouse amyloid-β peptide, although titres were generally an order of magnitude lower. Mice immunized with SAP peptides had antibody titres against SAP ranging from 1:1,000 to 1:10,000, with the exception of one mouse which had a titre greater than 1:10,000 (data not shown). Control animals receiving adjuvant alone had negligible titres to $A\beta_{42}$. At 13 months of age, quantitative immunohistochemical measures determined the extent of amyloid-β burden and the prevalence of neuritic plaques, astrogliosis and microgliosis.

Immunization with $A\beta_{42}$ resulted in almost complete prevention of amyloid-β deposition (Figs 1, 2). Seven of nine mice immunized with $A\beta_{42}$, including the mouse with the lowest anti-Aβ titre, had no detectable amyloid-β deposits in their brains. One mouse from this treatment group had a single isolated plaque in the six brain sections examined, whereas a second animal had a greatly reduced amyloid-β burden. Quantitative imaging of the amyloid-β burden in the hippocampus verified the near-total reduction achieved in $A\beta_{42}$-treated animals (Fig. 1). The median values of the amyloid-β burden for the PBS group (2.22%) and for the untreated control group (2.65%) were significantly greater than that of the Aβ-immunized group (0.00%, P 0.0005). In contrast, the median value of amyloid-β for the group immunized with SAP peptides (5.74%), although increased, was not significantly different from that of control animals. Brain tissue from the PBS-treated control mice contained numerous amyloid-β deposits in the hippocampus (Fig. 2) and retrosplenial cortex (not shown). We observed a similar pattern of amyloid-β deposition in mice immunized with SAP peptides. Brain sections from $A\beta_{42}$-immunized mice were also stained with thioflavin S to rule out the possibility that the lack of immunohistochemically detectable plaques was due to competition by the *de novo* anti-Aβ antibodies produced by these animals (see below). Thioflavin S detected no amyloid-β deposits in these $A\beta_{42}$-immunized mice (not shown). In contrast, immunization with SAP peptides did not affect amyloid-β deposition, suggesting that

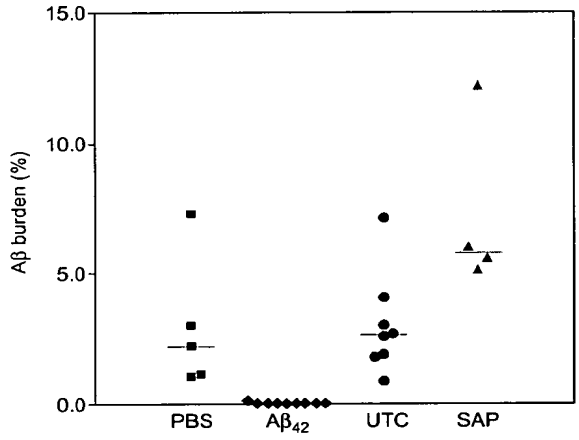

Figure 1 Reduction of Aβ burden in the hippocampus at 13 months of age in mice immunized with $A\beta_{42}$. PDAPP mice were immunized beginning at 6 weeks of age as described in Methods. The percentage of the area of the hippocampal region occupied by Aβ deposits was determined by quantitative image analysis. Values for individual mice are shown sorted by treatment group. Horizontal lines represent the median values. The $A\beta_{42}$-immunized group had significantly fewer Aβ deposits than any of the other three groups (P 0.001), which are not significantly different from each other ($P > 0.05$). UTC, untreated controls; SAP, mice immunized with serum amyloid P.

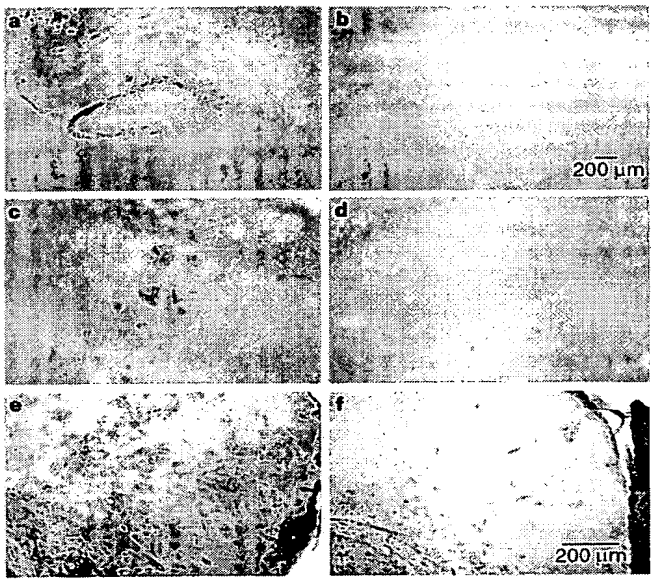

Figure 2 Hippocampal Aβ deposition, neuritic plaque formation and cortical astrocytosis in PBS- and $A\beta_{42}$-injected mice. Images from 13-month-old mice with Aβ burdens representative of the median values (see Fig. 1) of their respective groups are shown. **a**, **b**, Hippocampal Aβ plaques in PBS- (**a**) and $A\beta_{42}$-injected (**b**) mice. **a** shows abundant Aβ deposition in the outer molecular layer of the hippocampal dentate gyrus of a PBS-treated animal. **b** shows no detectable Aβ in this region of an $A\beta_{42}$-immunized mouse, a profile observed in most animals from this group. Scale bar in **b** corresponds to both **a** and **b**. **c**, **d**, Hippocampal sections from PBS- (**c**) and $A\beta_{42}$-injected (**d**) mice. Typical dystrophic neurites associated with neuritic plaques and labelled with the APP-specific monoclonal antibody 8E5 (ref. 16) were found in PBS- (**c**), but not $A\beta_{42}$-injected animals (**d**). **e**, **f**, Abundant plaque-associated astrocytosis, as determined by GFAP immunohistochemistry (Sigma), was evident in the retrosplenial cortex of PBS- (**e**) but not $A\beta_{42}$-injected (**f**) mice. Scale bar in **f** corresponds to **c**–**f**.

immune responses against plaque components *per se* are insufficient to prevent or eliminate β-amyloid plaques and neuropathology.

The brains of $A\beta_{42}$-treated mice that contained no amyloid-β deposits were also devoid of the dystrophic neurites that characterize the neuritic plaques (Fig. 2: compare c and d). Small numbers of dystrophic neurites were present in the two $A\beta_{42}$-treated mice that had detectable Aβ deposits. In contrast, all brains from SAP-injected mice and the two control groups (PBS-injected and untreated mice) had numerous neuritic plaques. Image analyses of the hippocampus demonstrated the virtual elimination of dystrophic neurites in the $A\beta_{42}$-treated mice (median, 0.00%) compared with the PBS recipients (median, 0.28%; P 0.0005).

Astrocytosis, another hallmark of plaque-associated pathology in both Alzheimer's disease and PDAPP mice, was dramatically reduced in the brains of all of the $A\beta_{42}$-injected mice (Fig. 2). Brains from mice in all other groups contained numerous clusters of astrocytes that were immunoreactive to glial fibrillary acidic protein (GFAP), a finding typical of Aβ-plaque-associated gliosis (Fig. 2e).

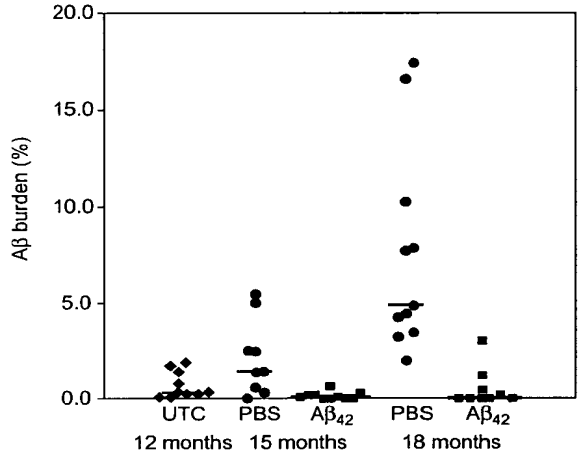

Figure 3 Quantitative image analysis of the cortical Aβ burden in older PBS- and Aβ-treated mice. Immunization of PDAPP mice was begun at 11 months of age. Amyloid burden was significantly reduced in the $A\beta_{42}$ group compared with the PBS controls at both 15 (P 0.003) and 18 (P 0.0002) months of age. The median value of the amyloid burden for each group is shown by the horizontal lines.

Association of amyloid plaques and reactive astrocytes was verified in a subset of GFAP-reacted sections counterstained with thioflavin S. The results of image analyses for the retrosplenial cortex verified that the reduction in astrocytosis was significant, with a median value of 1.55% for mice immunized with $A\beta_{42}$ compared with median values of greater than 6% for groups immunized with SAP peptide or PBS, or untreated control mice (P 0.0017).

Sections of the mouse brains were also reacted with a monoclonal antibody specific for MAC-1 (CD11b; Chemicon), a cell-surface marker that is upregulated on activated, plaque-associated microglia. MAC-1 labelling was substantially lower in the brains of mice treated with $A\beta_{42}$ compared with the PBS control group, a finding consistent with the lack of a plaque-induced gliosis (not shown).

The almost complete absence of plaques in the brains of $A\beta_{42}$-treated mice indicates that a fundamental mechanism of amyloid plaque formation has been disrupted. Subsequent studies indicate that Aβ production itself was unaffected (data not shown). $A\beta_{42}$ immunization therefore either prevents deposition and/or enhances the clearance of Aβ from the brain. The absence of neuritic and gliotic changes suggests that the $A\beta_{42}$-immunized mice never developed the neurodegenerative lesions that typify the progression of AD-like pathology in this model. The absence of enhanced astrocytosis, in particular, suggests that the processes preventing β-amyloidosis do not in themselves cause appreciable damage to the neuropil.

The above results clearly indicate that $A\beta_{42}$ immunization essentially prevents the development of AD-like neuropathology in the PDAPP mouse. It was unclear whether $A\beta_{42}$ immunization would improve the pathological outcome if treatment were initiated when a substantial Aβ plaque burden already existed. We therefore undertook further experiments in which immunizations with $A\beta_{42}$ began at an age when many β-amyloid plaques are already present in the brains of the PDAPP mice. Immunizations were continued during a period when the extent of Aβ deposition reaches levels comparable to those of established AD[11,12]. For this study, approximately 11-month-old, heterozygotic female PDAPP mice (n 24) were immunized repeatedly with $A\beta_{42}$ plus adjuvant, with an injection protocol and schedule equivalent to those used in the young PDAPP mouse study (see Methods). Similar titre responses against $A\beta_{42}$ to those seen in younger animals were generated in the older animals. As a negative control, a parallel group of 24 transgenic littermates was immunized with PBS plus adjuvant. One-half of each group was killed at 15 months of age after 4 months' treatment, and the remaining half was killed at 18 months

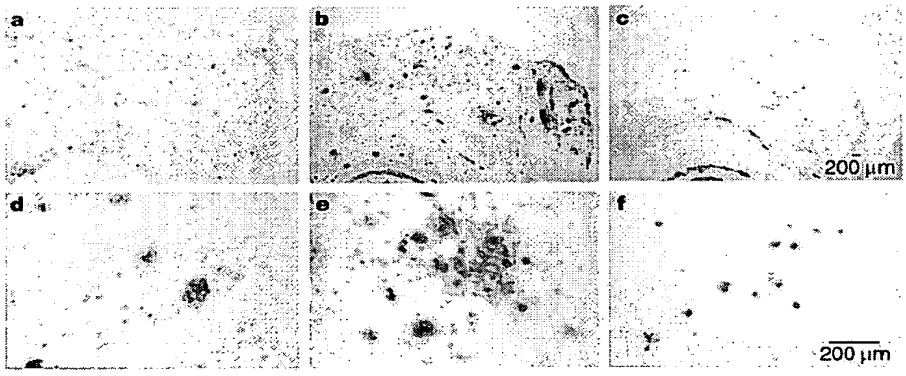

Figure 4 Reduction of cortical Aβ deposition in older PDAPP mice immunized with $A\beta_{42}$. Aβ deposits in the brains of 12-month-old untreated PDAPP mice and 18-month-old PBS- and $A\beta_{42}$-injected mice with median Aβ burdens representative of their respective treatment groups (see Fig. 3). **a**, Distribution of amyloid plaques in the frontal and retrosplenial cortices of a 12-month-old untreated PDAPP mouse, typifying the plaque burden of both PBS- and $A\beta_{42}$-injected groups at the start of the study. The plaques are shown at a high magnification in **d**. Compared with the 18-month PBS controls (**b**, **e**), Aβ deposits were significantly decreased in the 18-month $A\beta_{42}$-immunized group (**c**, **f**). Most of the cortical Aβ in brains of $A\beta_{42}$-injected mice was detected in small extracellular or cell-associated deposits (**f**) compared with the large and numerous extracellular deposits in the PBS group (**e**). Scale bar in **c** corresponds to **a**–**c**. Scale bar in **f** corresponds to **d**–**f**.

of age after 7 months' treatment. Groups of untreated PDAPP mice were also killed at ages 12, 15 and 18 months to serve as age-matched, non-immunized controls. At each time point, brains were examined by image analysis and enzyme-linked immunosorbent assay (ELISA) to determine the magnitude of the amyloid-β burden and the extent of neuritic dystrophy, astrocytosis and microgliosis.

Figure 3 shows the results of $A\beta_{42}$ treatment on cortical amyloid-β burden, determined by quantitative image analysis. The median value of cortical amyloid-β burden was 0.28% in untreated, 12-month-old PDAPP mice, a value representative of the plaque load in the experimental mice at the start of the study. At 18 months, the amyloid-β burden had increased by more than 17-fold to 4.87% in PBS-treated mice, while $A\beta_{42}$-treated mice had a greatly reduced amyloid burden of only 0.01%. The amyloid-β burden was significantly reduced in the $A\beta_{42}$ recipients at both 15 months (96% reduction; P 0.003) and 18 months (>99% reduction; P 0.0002). Figure 4 depicts the burden of amyloid-β in the 12-month-old PDAPP brain at the start of the experiment (Fig. 4a, d). At 18 months of age, the progression of Aβ-plaque pathology was obvious in the PBS group (Fig. 4b, e), but greatly diminished in the $A\beta_{42}$-injected mice (Fig. 4c, f). Compared with the 12-month-old untreated mice, several brains from the $A\beta_{42}$ group had fewer diffuse and mature amyloid-β deposits at 15 and 18 months, suggesting that the treatment had resulted in the clearance of pre-existing amyloid-β deposits (Fig. 4: compare a and d with c and f). Immunohistochemistry with anti-mouse immunoglobulin antibodies showed that the remaining plaques were often decorated with IgG in $A\beta_{42}$-treated but not in PBS-treated mice at both 15 and 18 months of age (data not shown).

Cortical amyloid-β deposition in PDAPP brains begins in the cingulate, frontal and retrosplenial cortices and progresses in a lateral–ventral fashion to sequentially involve the temporal and entorhinal cortices. After 3 months of treatment, amyloid-plaque pathology was diminished in the retrosplenial cortex (Fig. 5a, c) and completely absent in the entorhinal cortex (Fig. 5b, d) of the $A\beta_{42}$-injected mice. The progressive β-amyloidosis that would normally pervade the entorhinal cortex was thus halted by $A\beta_{42}$ immunization.

PDAPP mice also invariably develop heavy amyloid-β deposition in the outer molecular layer of the hippocampal dentate gyrus[11]. In a number of brains from $A\beta_{42}$-immunized mice, this pattern was considerably altered; the hippocampal deposition no longer contained diffuse amyloid-β deposits, and the banded pattern was completely disrupted (Fig. 6). Instead, unusual punctate structures were present that were reactive with anti-Aβ antibodies, and several appeared to be Aβ-containing cells (Fig. 6b). The pattern of apparent cellular labelling produced by the amyloid-β antibodies was replicated in adjacent sections by immunolabelling with antibodies directed at major histocompatibility complex (MHC) class II molecules (Fig. 6c). Phenotypically, these cells resembled activated microglia and monocytes and were occasionally found associated with the wall or lumen of blood vessels. They were not immunoreactive with antibodies that recognize T-cell (CD43, CD3e) or B-cell (CD45RA, CD45RB) surface markers (data not shown). No such cells were found in any of the PBS-treated mice.

Amyloid-β ELISA analysis of the older PDAPP mice was consistent with the immunohistochemical observations. In untreated PDAPP mice, the median level of total Aβ in the cortex at 12 months was 1,600 ng per g (wet weight); this had increased to 8,700 ng per g by 15 months[12]. At 18 months the value was 22,000 ng per g, an increase of more than 10-fold during the course of the experiment[12]. PBS-treated animals had comparable levels of total amyloid-β at 15 months (8,600 ng per g), and 19,000 ng per g at 18 months. In contrast, $A\beta_{42}$-treated animals had 81% less total amyloid-β at 15 months (1,600 ng per g) than the PBS-immunized group. Significantly less total amyloid-β (5,200 ng per g) was found at 18 months when the $A\beta_{42}$ and PBS groups were compared, representing a 72% reduction (P 0.0001) in the amyloid-β that would have otherwise been present. Similar results were obtained when cortical levels of $A\beta_{42}$ were compared, namely that the $A\beta_{42}$-treated group contained much less $A\beta_{42}$, and the differences between the $A\beta_{42}$ and PBS groups were significant at 15 months (P 0.04) and 18 months (P 0.0001). In contrast, cortical levels of APP decreased by less than 10% of those in 12-month-old untreated animals (data not shown)[12]. These findings argue against the possibility that the reduction in amyloid-β deposition seen in the treated mice is due to an alteration in APP metabolism.

The progression of neuritic pathology was significantly reduced in the frontal cortex of $A\beta_{42}$-treated compared with PBS-treated mice (Table 1). At 15 months of age, the neuritic plaque burden in

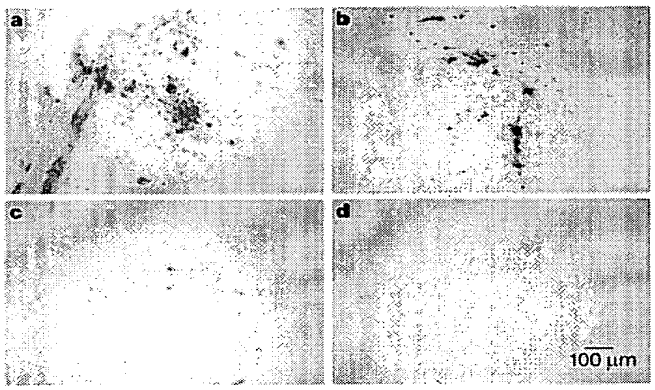

Figure 5 Reduction of Aβ burden in the entorhinal and retrosplenial cortex of older PDAPP mice following Aβ injection. Aβ deposition in the retrosplenial (RSC) and entorhinal (EC) cortices of 15-month-old PBS- (**a**, **b**) and $A\beta_{42}$- (**c**, **d**) injected mice with Aβ burdens representative of the median values of their respective groups. Aβ deposition was greatly reduced in the RSC of $A\beta_{42}$-injected mice compared with the PBS group (compare **a** and **c**). No Aβ was detected in the EC of $A\beta_{42}$-injected mice (**d**), in contrast to the PBS group (**b**). Scale bar in **d** corresponds to all panels.

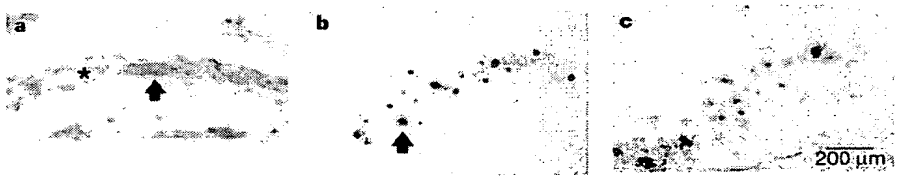

Figure 6 Altered Aβ burden in the hippocampus of older $A\beta_{42}$-treated mice. Distribution of hippocampal Aβ in $A\beta_{42}$-injected brains (**b**), compared with the PBS group (**a**) at 18 months of age. In the PBS group, the characteristic appearance of diffuse (asterisk) and compacted (arrow) Aβ deposits was evident (**a**). This pattern was markedly altered in a number of $A\beta_{42}$-immunized mice (**b**), with the absence of diffuse deposits and an unusual punctate pattern of Aβ immunoreactivity associated with cells (**b**, arrow). The distribution of MHC II-labelled (Pharmigen) cells in a near-adjacent section (**c**) corresponded to the pattern of Aβ-positive cellular labelling shown in **b**. No such obvious cell Aβ staining was found in the PBS group (compare **a** and **b**). Scale bar in **c** corresponds to all panels.

Table 1 Image analysis of neuritic plaque and astrocytic burden in $A\beta_{42}$-treated PDAPP mice

	15 months		18 months	
	PBS	$A\beta_{42}$-treated	PBS	$A\beta_{42}$-treated
Neuritic plaque (%)	0.32	0.02	0.49	0.22
Astrocytosis (%)	4.26	1.89	5.21	3.20

Quantitative image analysis of neuritic plaques and astrocytosis was performed using antibody 8E5 to human APP and anti-GFAP, respectively. Methods are described in Fig. 1 legend. Reduction of neuritic plaque burden at ages 15 and 18 months by $A\beta_{42}$ treatment was statistically significant (P 0 03 and 0.01, respectively), as was reduction of astrocytosis at the same time points (P 0 01 and 0.03, respectively).

the $A\beta_{42}$-treated mice was reduced by 84% compared with the PBS group (0.05% and 0.32%; P 0.03). Reduction in the neuritic-plaque pathology was similarly maintained between the two groups at 18 months of age, where the degree of neuritic dystrophy was reduced by 55% in the $A\beta_{42}$-treated mice (0.22% and 0.49%; P 0.01).

Reactive astrocytosis was also significantly reduced in the retrosplenial cortex of $A\beta_{42}$-treated mice compared with PBS-treated mice at both 15 and 18 months of age. The per cent of astrocytosis in the PBS group increased between 15 and 18 months from 4.29% to 5.21%. $A\beta_{42}$ treatment suppressed the development of astrocytosis at both time points to 1.89% and 3.2%, respectively. These differences represent a 56% reduction (P 0.01) at 15 months of age and a 34% decrease (P 0.03) at 18 months of age in the $A\beta_{42}$-treated group.

In summary, immunization with $A\beta_{42}$ greatly reduced the development of the AD-like pathology that otherwise occurs in the PDAPP mouse. Immunization preceding plaque development profoundly affected the occurrence of new lesions, as the progression of β-amyloidosis and associated neuropathology was essentially wholly blocked, as seen both in the entire brain of the young animals and in at-risk brain regions of the older animals. Amyloid-β immunization also significantly retarded the progression of existing pathology in affected regions of the older animals. Outcomes of Aβ-plaque burden, neuritic dystrophy and gliosis were all significantly improved by $A\beta_{42}$ treatment in both young and old animals. In addition, the mechanism resulting in plaque reduction did not seem to produce any obvious signs of damage to the neuropil of $A\beta_{42}$-immunized animals. Histological examination of several organs, including brain and kidney, revealed no signs of immune-mediated complications, despite the high levels of human APP expressed in these tissues and the significant antibody titre to endogenous mouse Aβ peptide (data not shown).

To our knowledge, this is the first report of a clinically relevant treatment that reduces the progression of AD-like neuropathology in a transgenic animal model of the disease. Although it remains unproven, it is not unreasonable to expect that a similar reduction of neuropathology in AD patients would be of clinical benefit. Although our understanding of the precise aspects of the immune response that result in reduced pathology is incomplete, we have shown that $A\beta_{42}$ immunization results in the generation of anti-Aβ antibodies and that Aβ-immunoreactive monocytic/microglial cells appear in the regions of remaining plaques. Thus, one possible mechanism of action is that anti-Aβ antibodies facilitate clearance of amyloid-β either before deposition, or after plaque formation, by triggering monocytic/microglial cells to clear amyloid-β using signals mediated by F_c receptors.

It has been suggested that a chronic inflammatory state exists in the brain of patients with Alzheimer's disease: specifically the levels of complement, cytokines and acute-phase proteins are raised[14]. These observations have led to the hypothesis that anti-inflammatory regimens might be of therapeutic value. The findings presented here argue that an alternative approach, one that augments a highly specific immune response, can markedly reduce pathology in an animal model of the disease. Collectively, the results suggest that amyloid-β immunization may prove beneficial for both the treatment and prevention of Alzheimer's disease. □

Methods

Immunization procedures. Aβ peptide was freshly prepared from lyophilized powder for each set of injections. For immunizations, 2 mg $A\beta_{42}$ (human $A\beta_{1-42}$; US Peptides) was added to 0.9 ml deionized water and the mixture was vortexed to generate a relatively uniform suspension. A 100-μl aliquot of 10 × PBS (where 1 × PBS is 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was added. The suspension was vortexed again and incubated overnight at 37 °C for use the next day. Serum amyloid-P component immunogens were prepared using mouse SAP amino acids 77–85 and 164–173, each conjugated to sheep anti-mouse IgG (Jackson Immunochemicals) as described[15]. $A\beta_{42}$ or SAP peptides (100 μg antigen per injection) were emulsified 1:1 (v/v) with complete Freund's adjuvant for the first immunization, followed by a boost in incomplete Freund's adjuvant at 2 weeks, and monthly thereafter. $A\beta_{42}$ or SAP in PBS alone was injected from the fifth immunization onward. Titres were determined by serial dilutions of sera against either aggregated $A\beta_{42}$ or SAP protein which had been coated onto microtitre wells. Detection used goat anti-mouse immunoglobulin conjugated to horseradish peroxidase and slow-TMB (3,3″,5,5′-tetramethyl benzidine; Pierce) substrate. Titres were defined as the dilution yielding 50% of the maximal signal.

Neuropathology quantification. To quantify amyloid burden, PDAPP mouse brain tissue was fixed in 4% paraformaldehyde, cut to 40-μm coronal sections and reacted with an anti-Aβ biotinylated antibody, 3D6, as described previously[11]. Quantitative image analysis was performed using a Videometric 150 Image Analysis System (Oncor) linked to a Nikon Microphot-FX microscope through a CCD video camera. The image of the immunoreacted section was stored in a video buffer and a specific brain region (hippocampus or cortex) was manually outlined and the total pixel area occupied by the structure determined. A monochromatic-based threshold was set to select pixels corresponding to immunolabelled structures. The per cent of the brain region occupied by the labelled pixels was then calculated. For all image analyses, six sections at the level of the dorsal hippocampus, each separated by consecutive 240-μm intervals, were evaluated for each animal. In all cases, the treatment status of the animals was unknown to the observer. Mann–Whitney nonparametric analysis was performed using Statview software (SAS Institute, Cary, NC). Similar methodologies were used to quantify neuritic dystrophy and gliosis. Specific reagents are indicated in the appropriate figures.

Received 6 April; accepted 5 May 1999.

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Acknowledgements. We thank Rae Lyn Burke for helpful comments.

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Data analysis

Results are expressed as mean ± s.e.m. of n separate experiments. The significance of differences among groups was evaluated using ANOVA followed by a Student–Newman–Keuls *post-hoc* test, unless indicated otherwise.

Received 5 June; accepted 12 September 2001.

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Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

Acknowledgements

We thank M. Schwartz for comments; R. Carrera and A. M. Basso for help with initial experiments; T. Dinh, M. Guzmán and C. Sánchez for reading the manuscript critically; M. A. Gorriti, J. Muñoz, F. Désarnaud, M. A. Villanúa and Y. Xie for help with experiments; and F. Valiño for editorial assistance. This research was supported by grants from the National Institute of Drug Abuse (to D.P.) and from the National Institute of Mental Health (to C.G.). Additional support was from the Comunidad de Madrid, Del Amo Program and Plan Nacional sobre Drogas (to F.R.F. and M.N.). F.R.F. is a research fellow of the Jaime del Amo Program, Complutense University.

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A subset of NSAIDs lower amyloidogenic Aβ42 independently of cyclooxygenase activity

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Epidemiological studies have documented a reduced prevalence of Alzheimer's disease among users of nonsteroidal anti-inflammatory drugs (NSAIDs)[1–5]. It has been proposed that NSAIDs exert their beneficial effects in part by reducing neurotoxic inflammatory responses in the brain, although this mechanism has not been proved. Here we report that the NSAIDs ibuprofen, indomethacin and sulindac sulphide preferentially decrease the highly amyloidogenic Aβ42 peptide (the 42-residue isoform of the amyloid-β peptide) produced from a variety of cultured cells by as much as 80%. This effect was not seen in all NSAIDs and seems not to be mediated by inhibition of cyclooxygenase (COX) activity, the principal pharmacological target of NSAIDs[6]. Furthermore, short-term administration of ibuprofen to mice that produce mutant β-amyloid precursor protein (APP) lowered their brain levels of Aβ42. In cultured cells, the decrease in Aβ42 secretion was accompanied by an increase in the Aβ(1–38) isoform, indicating that NSAIDs subtly alter γ-secretase activity without significantly perturbing other APP processing pathways or Notch cleavage. Our findings suggest that NSAIDs directly affect amyloid pathology in the brain by reducing Aβ42 peptide levels independently of COX activity and that this Aβ42-lowering activity could be optimized to selectively target the pathogenic Aβ42 species.

Increasing evidence suggests that a key event in the pathogenesis of Alzheimer's disease is the altered production, aggregation and deposition of the Aβ peptide, a proteolytic fragment of 40–42 residues derived from APP. The longer isoform, Aβ42, is selectively increased in all presenilin mutations analysed and in most APP mutations that cause early-onset familial Alzheimer's disease. Aβ42 is the Aβ species initially deposited in brain, and is particularly prone to aggregation *in vitro*. Therefore, Aβ42 is believed by many to be the main culprit in the pathogenesis of Alzheimer's disease[7].

We examined whether NSAIDs alter APP processing and generation of Aβ, particularly the Aβ42 species. We treated cells with increasing concentrations of various NSAIDs, and analysed Aβ40 and Aβ42 levels in culture medium by sensitive sandwich enzyme-link immunosorbent assay (ELISA) as described previously[8]. The range of NSAID concentrations was chosen on the basis of tolerated plasma concentrations achieved in humans[9]. Multiple NSAIDs were examined in this study, several of them sharing similar activities (see below; an overview describing the cell lines and compounds studied is provided in the Supplementary Information). For brevity, the non-selective COX-inhibitor sulindac sulphide, the active metabolite of the pro-drug sulindac with well recognized antineoplastic

properties[10], is presented as the prototypic NSAID in its striking preferential inhibition of Aβ42 secretion. In Chinese hamster ovary (CHO) cells stably transfected with both APP751 (wild-type APP, WT-APP) and the PS1 mutant M146L (PS1-M146L), a 50–70% reduction in the Aβ42/Aβ40 quotient was achieved at concentrations of 40–60 μM without significant reduction in total Aβ (Aβ40 + Aβ42) levels (Fig. 1a). This result was confirmed in CHO cells overexpressing WT-APP and in CHO cells transfected with the APP V717F mutation (see Supplementary Information). To exclude the possibility that this effect is specific to a particular cell type, we examined Aβ secretion in response to sulindac sulphide treatment in the human neuroglioma cell line HS683 stably transfected with APP695. A dose-dependent inhibition of Aβ42 secretion similar to that seen with CHO cells was observed (Fig. 1b). Sulindac sulphide also reduced Aβ42 secretion in kidney HEK293 cells, H4 neuroglioma cells, and primary mouse embryonic fibroblasts (Supplementary Information). Thus, this effect was observed in cell lines of rodent and human origin and is not dependent on cell type.

Ibuprofen reduced amyloid plaque pathology in a mouse model of Alzheimer's disease, and indomethacin seemed to slow the cognitive decline in patients with Alzheimer's disease in a placebo-controlled pilot study[3,11]. These two non-selective COX inhibitors also decreased the Aβ42/Aβ40 quotient by selective inhibition of Aβ42 secretion in a dose-dependent manner. In WT-APP PS1-M146L CHO cells, a 50% reduction in the Aβ42/Aβ40 quotient was reached at ibuprofen concentrations of 200–300 μM (Fig. 1c) and at indomethacin concentrations of 25–50 μM (Fig. 1d). Similarly, total Aβ levels were not significantly affected with either ibuprofen (up to 500 μM) or indomethacin (up to 150 μM) (Fig. 1c and d). No toxicity was detected by standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay in CHO or HS683 cells or by [^{3}H]-thymidine incorporation for CHO cells treated with sulindac sulphide concentrations up to 100 μM, with indomethacin up to 200 μM, or with ibuprofen up to 1 mM (data not shown). Therefore, the Aβ42 effect is not related to cytotoxicity.

Table 1 Acute dosing with ibuprofen lowers brain Aβ42

Treatment	*n*	Aβ40 (pmol g^{-1})	Aβ42 (pmol g^{-1})	Aβ42 (% of Aβ40 + Aβ42)
Control	18	18.5 ± 2.8	7.7 ± 1.3	29.5 ± 3.4
Naproxen	7	18.6 ± 1.1	7.9 ± 0.6	29.8 ± 1.6
Ibuprofen	15	17.5 ± 1.7	4.7 ± 2.0*	20.8 ± 7.5*

Tg2576 mice were dosed as described in the text. Values are expressed as mean ± s.d.
*$P < 0.01$ by Dunnet's test.

We next examined sulindac sulphone, a second oxidative metabolite of sulindac with potent antineoplastic effects that lacks COX-inhibitory activity[12]. Unlike sulindac sulphide, sulindac sulphone had no effect on the Aβ42/Aβ40 quotient in WT-APP CHO cells with concentrations up to 400 μM (data not shown). This finding suggests a role for COX inhibition in the preferential reduction of Aβ42 secretion by NSAIDs. We therefore investigated whether reduction of Aβ42 levels is a characteristic common to all NSAIDs by assessing several other clinically approved NSAIDs. Naproxen is a non-selective COX inhibitor that belongs to the same structural class as ibuprofen. However, unlike ibuprofen or sulindac sulphide, treatment of WT-APP CHO cells with naproxen at concentrations up to 400 μM did not lower either the Aβ42/Aβ40 quotient or total Aβ levels (Fig. 1e). Similar negative results were obtained with aspirin, meloxicam (an NSAID with preferential COX-2 activity), SC-560 (a selective COX-1 inhibitor) and celecoxib (a selective COX-2 inhibitor) (see Supplementary Information). These data demonstrated that the capacity to lower Aβ42 secretion is not associated with all NSAIDs. Furthermore, the effective NSAID concentrations used in our experiments were clearly in excess of the levels required for complete inhibition of COX-1 and COX-2 in cell-based assays[13] (see Supplementary Information), indicating that the reduction in Aβ42 levels may be independent of COX activity.

To demonstrate that the decrease in Aβ42 levels is not mediated by inhibition of COX activity and concomitant reduction in prostaglandin synthesis, fibroblasts deficient in both COX-1 and COX-2 by targeted gene deletions (COX-1$^{-/-}$ COX-2$^{-/-}$)[14] (see Supplementary Information) were examined for the effect of NSAIDs on Aβ42 levels. The basal levels of Aβ peptides were unchanged compared with littermate control mouse fibroblasts, indicating that the loss of COX-1 and COX-2 enzymes did not by itself alter Aβ42 levels (data not shown). However, treatment with sulindac sulphide reduced Aβ42 levels and altered the Aβ42/Aβ40 quotient in a similar fashion to that seen in CHO and HS683 neuroglioma cells (Fig. 1f) and identical to littermate control

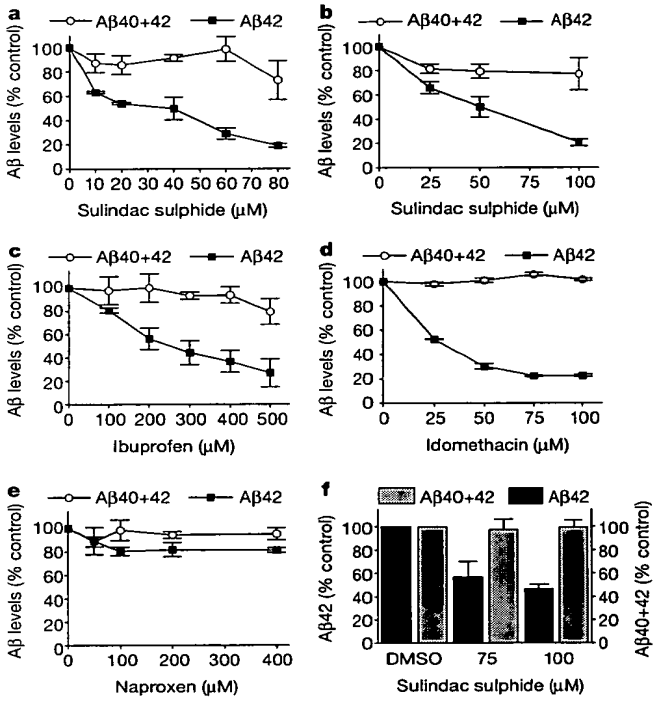

Figure 1 Analysis of Aβ from cultured cells treated with NSAIDs by ELISA. Aβ42 and total Aβ levels (Aβ40 plus Aβ42 values, Aβ40+42) were normalized to values obtained from vehicle-treated cells. Treatment with sulindac sulphide preferentially reduced Aβ42 levels in the medium of WT-APP PS1-M146L CHO cells (**a**) and human neuroglioma cells (**b**) in a dose-dependent manner. Selective inhibition of Aβ42 levels was also observed with ibuprofen (**c**) and indomethacin (**d**), but not with naproxen (**e**) in WT-APP CHO cells. Sulindac sulphide similarly reduced Aβ42 levels in fibroblasts deficient in COX-1 and COX-2 (**f**). Each panel shows the mean ± s.d. of all experiments.

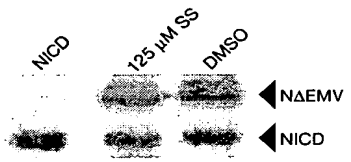

Figure 2 Analysis of Notch processing after treatment with sulindac sulphide in HEK 293 cells. NICD formation from a constitutively cleaved Notch construct (NΔEMV) was not impaired in cells treated with sulindac sulphide (125 μM SS). The left lane shows control transfection with an NICD construct to identify the cleavage fragment.

fibroblasts (data not shown). These results provide compelling evidence that the reduction in Aβ42 is not mediated by inhibition of COX activity, the principal mode of action of NSAIDs. Whether other NSAID-responsive pathways mediate this activity, such as by modulating lipoxygenase activity or transcription controlled by peroxisome proliferator-activated receptors (PPARs), is unknown.

In a recent study, chronic high-dose ibuprofen treatment was shown to significantly reduce amyloid pathology, neuritic dystrophy, plaque-associated gliosis and IL-1 expression in Tg2576 transgenic mice[11]. After 6 months of treatment, amyloid plaque numbers and Aβ levels in brain were reduced almost 50% and 40%, respectively. To determine whether the COX-independent reduction in Aβ42 seen with some NSAIDs in cultured cells could in part account for the long-term effects that have been reported, we examined whether treatment of Tg2576 mice with ibuprofen preferentially reduces Aβ42 levels. For these studies, 3-month-old female Tg2576 mice were orally dosed with 50 mg kg^{-1} d^{-1} of ibuprofen (n = 15) or 50 mg kg^{-1} d^{-1} of naproxen (n = 7), or mock treated (n = 18), and brain levels of SDS-soluble Aβ40 and Aβ42 were measured by ELISA. Administration of ibuprofen resulted in a highly significant 39% decrease in levels of Aβ42 without any changes in Aβ40 compared with mock-treated animals (Table 1 and Supplementary Information). Consistent with the tissue culture results, treatment with naproxen did not alter the levels of Aβ42 in brain at a dose that has been shown to inhibit prostaglandin production in rodent brain[15]. These results strongly indicate the physiological relevance of our *in vitro* studies. In the previous study[11], a reduction in parenchymal but not vascular amyloid deposits was noted. Because vascular Aβ deposits consist primarily of Aβ40, whereas parenchymal deposits are a mixture of Aβ40 and Aβ42, the lack of effect on vascular amyloid is in agreement with our hypothesis that ibuprofen treatment may prevent amyloid pathology by decreasing the Aβ42/Aβ40 quotient in brain.

NSAIDs are unique in their ability to change the Aβ42/Aβ40 quotient by selectively decreasing Aβ42 levels. Recently, γ-secretase inhibitors have been developed that either inhibit the generation of all Aβ species or show preferential activity against Aβ40. Several of these inhibitors have been demonstrated to bind presenilins, leading to the hypothesis that presenilins are actual γ-secretases[16]. Because presenilins are essential not only for γ-secretase cleavage of APP but also for proteolytic processing of the Notch receptor[17], γ-secretase inhibitors may concomitantly inhibit Notch cleavage, resulting in adverse side effects[18]. Furthermore, treatment with γ-secretase inhibitors leads to the accumulation of high amounts of carboxy-terminal fragments (CTFs) of APP that may be neurotoxic[19,20]. We therefore examined multiple parameters in APP processing as well as Notch intramembrane cleavage to determine whether any of these cellular pathways are altered in response to NSAID treatment. WT-APP CHO cells were treated with increasing concentrations of sulindac sulphide as described above. Western blot analysis with an APP C-terminal polyclonal antibody did not show any changes in the levels of full-length APP or APP CTF species cleaved by α- or β-secretase (see Supplementary Information). Similarly, secretion of

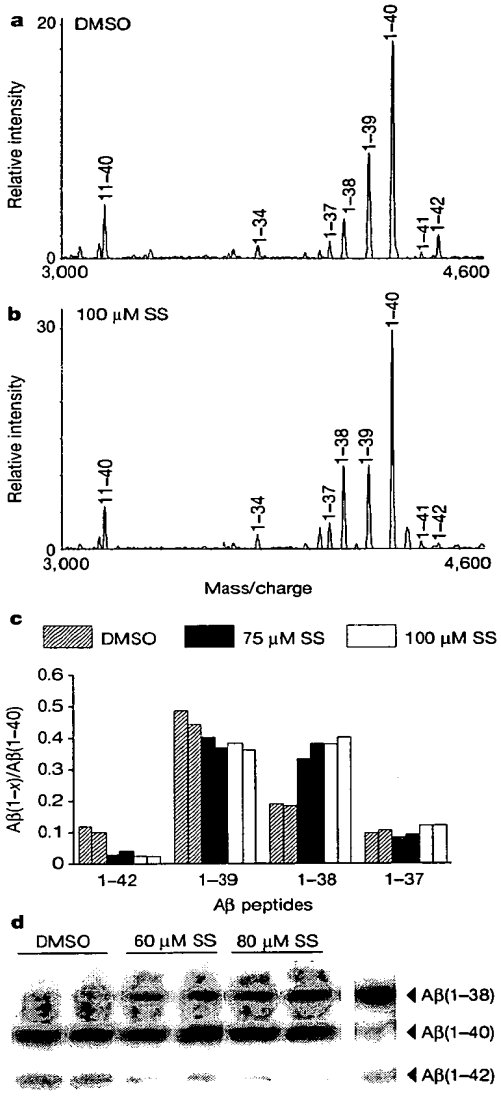

Figure 3 Aβ species in medium after NSAID treatment. **a**, Representative mass spectra of Aβ peptides from WT-APP CHO cells treated with 100 μM sulindac sulphide (SS) or DMSO vehicle showing a decrease in Aβ(1–42) species but an increase in Aβ(1–38) species. **b**, Quantitative mass spectrometry analysis of Aβ peptide levels calculated as a quotient, Aβ(1–x)/Aβ(1–40). **c**, The changes in Aβ(1–42) and Aβ(1–38) levels in medium were confirmed by bicine/urea SDS–PAGE. Standard Aβ(1–38), Aβ(1–40) and Aβ(1–42) peptides were used to identify Aβ species (right lane). The lane with control peptides was intentionally offset because the exposure time was longer to provide comparable band intensities. **d**, Reduction in Aβ(1–42)/Aβ(1–40) was accompanied by an increase in Aβ(1–38)/Aβ(1–40). Duplicate measurements are shown.

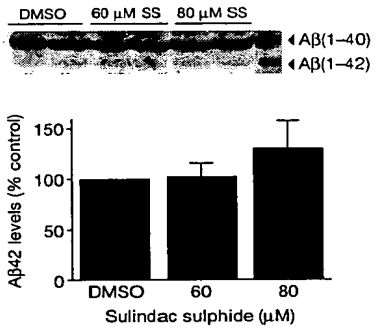

Figure 4 Turnover of Aβ42 after NSAID treatment. Aβ40 and Aβ42 were assayed from untransfected CHO cells incubated with conditioned medium obtained from CHO cells transfected with WT-APP and treated with increasing concentrations of sulindac sulphide (SS) (see Methods). The levels of Aβ40 and Aβ42 from the conditioned medium were virtually identical to vehicle-treated controls analysed by western blotting (top) or ELISA (bottom), indicating that Aβ turnover (clearance or degradation) was not affected by NSAID treatment. The ELISA results represent mean ± s.d. of all experiments.

the APP ectodomain (APPs) was not altered, as determined by western blotting analysis of conditioned medium using two different APP monoclonal antibodies (see Supplementary Information). Furthermore, by pulse-chase metabolic labelling, APP turnover was unchanged after treatment with sulindac sulphide (see Supplementary Information).

We next examined APP internalization because processing of APP in the endocytic pathway is hypothesized to be a major route for the generation and secretion of both Aβ40 and Aβ42 (ref. 21). Using a previously described APP internalization assay[21], we found the ratio of cell surface APP to internalized APP to be virtually identical in cells treated with sulindac sulphide compared with vehicle-treated cells (see Supplementary Information). Finally, we analysed Notch intramembrane cleavage and formation of the Notch intracellular cytoplasmic domain (NICD) in HEK293 cells after transfection with the Myc-tagged NΔEMV complementary DNA, an artificial Notch receptor construct that undergoes constitutive cleavage[22]. Consistent with the results for APP CTFs shown above, treatment with sulindac sulphide did not impair Notch cleavage or NICD formation (Fig. 2). Similarly, treatment with ibuprofen (500 μM) or indomethacin (150 μM) did not result in either accumulation of APP CTFs or inhibition of Notch cleavage (data not shown). These results demonstrate that NSAID treatment did not significantly perturb APP processing or γ-secretase activity. However, the preferential decrease in Aβ42 could be explained by minor changes in γ-secretase activity that were not detectable in the preceding assays.

To determine how NSAIDs may alter Aβ42 levels, two additional sets of experiments were performed. First, we analysed the full spectrum of Aβ species secreted by cells treated with sulindac sulphide by immunoprecipitation with mass spectrometry (IPMS). In contrast to ELISA measurements, IPMS provides definitive information about the length and identity of the individual Aβ peptides, for example Aβ(1–38), Aβ(1–40), and so on. The IPMS results extended our findings in significant ways. As expected, treatment with 75–100 μM sulindac sulphide reduced the level of Aβ(1–42) whereas Aβ(1–40) was essentially unaffected. Remarkably, the decrease in Aβ(1–42) secretion was accompanied by a dose-dependent increase in Aβ(1–38) species, resulting in a two-fold increase in the Aβ(1–38)/Aβ(1–40) quotient (Fig. 3a and b). Other Aβ peptide species (Aβ(1–37) and Aβ(1–39)) did not show consistent changes after treatment. To confirm the IPMS results, Aβ was immunoprecipitated from conditioned media of WT-APP PS1-M146L CHO cells and fractionated on a gel system that allows the resolution of individual Aβ species[23]. As anticipated, a large reduction in an immunoreactive band corresponding to Aβ(1–42) was accompanied by a dose-dependent increase in an immunoreactive species corresponding to Aβ(1–38) (Fig. 3c).

Two potential mechanisms may explain this unprecedented change in Aβ production after NSAID treatment. Sulindac sulphide and other NSAIDs could reduce Aβ42 secretion by shifting γ-secretase activity towards production of Aβ38. Alternatively, NSAIDs may stimulate the activity of an exopeptidase that converts Aβ42 into shorter Aβ species such as Aβ38. In the second experiment, we therefore asked whether the turnover of Aβ42 in culture medium could be enhanced after NSAID treatment. Up to this point, only the levels of Aβ peptides had been assessed and it is possible that NSAIDs may facilitate the degradation or cell-mediated clearance of Aβ42 (refs 24–26). To examine this possibility, untransfected CHO cells were cultured in conditioned medium obtained from WT-APP CHO cells supplemented with increasing concentrations of sulindac sulphide. Because untransfected CHO cells do not produce Aβ to any significant level, all the measurable Aβ peptides from this culture paradigm were derived exogenously. Under this condition, sulindac sulphide did not reduce the levels of Aβ42 compared with controls (Fig. 4), indicating that NSAIDs did not selectively target Aβ42 for catabolism, either by activating exopeptidase activity or by selective clearance. Future studies will be required to address this complex mechanism as there are no precedents for this pharmacological alteration in Aβ C-terminal cleavages, putatively attributable to altered γ-secretase activity.

Our observations suggest a mechanism through which NSAIDs might confer protection from Alzheimer's disease that is independent of direct anti-inflammatory properties. Because not all NSAIDs lower Aβ42 levels, this Aβ42 activity may be an important criterion to consider in clinical trials of NSAIDs for the treatment of Alzheimer's disease. Our results suggest an explanation for the variable data obtained from epidemiological studies and recent negative clinical results with COX-2-selective NSAIDs[27], as we find that many NSAIDs (including these selective COX-2 inhibitors) lack the ability to lower Aβ42. However, our results do not exclude the potential benefit of NSAIDs in reducing the inflammatory response in the Alzheimer's disease brain. Finally, in contrast to the current generation of γ-secretase inhibitors, NSAIDs do not perturb APP or Notch processing but rather seem to induce a subtle shift in γ-secretase activity. However, significant gastrointestinal and renal toxicity associated with long-term COX-1 inhibition limit the clinical utility of current NSAIDS as Aβ42-lowering agents. Because the Aβ42 effect we described is independent of COX activity, compounds with optimized Aβ42 reduction and little to no inhibition of COX-1 activity are likely to be identified. Such agents would represent a new generation of 'anti-amyloid' drugs that selectively target production of the highly amyloidogenic Aβ42 species without inhibiting either COX activity or the vital physiological functions of γ-secretase. □

Methods

Cell culture and drug treatment

CHO cells stably transfected with human APP751 or transfected with both human APP751 and human mutant PS1 (M146L), CHO cells transfected with human mutant APP751 (V717F), human neuroglioma cells HS683 transfected with APP695, HEK 293 cells transfected with APP695, and spontaneously immortalized embryonic fibroblasts from COX1- and COX-2-deficient animals were maintained in DMEM medium supplemented with 10% fetal bovine serum and 100 U ml^{-1} penicillin/streptomycin (Life Technologies). The NSAIDs sulindac sulphide (50 mM, Biomol), sulindac sulphone (50 mM, Biomol), naproxen (100 mM, Cayman Chemical), aspirin (2.5 M, ICN Biomedicals), meloxicam (50 mM, Calbiochem) and SC-560 (50 mM, Calbiochem) were dissolved in dimethyl sulphoxide (DMSO). Indomethacin (50 mM, Biomol) and (S)-ibuprofen (250 mM, Biomol) were dissolved in ethanol. Celecoxib was prepared from commercial Celebrex capsules (Searle) by solvent extraction followed by recrystallization. Nuclear magnetic resonance (NMR) and mass spectrometry verified the identity and purity of celecoxib. Purified celecoxib was dissolved in ethyl acetate (10 mM). For analysis of Aβ secretion, APP processing and Notch cleavage, cells were cultured in serum-containing medium and pretreated overnight with the specific NSAIDs. Medium was changed and treatment was continued for another 24 h. All experiments were repeated 2–4 times in duplicate or triplicate and results either from a representative experiment or from all experiments (mean ± s.d.) are shown.

ELISA

Aβ was analysed by sandwich ELISA as described previously[8]. Media were collected after conditioning for 24 h, and cell debris was removed by centrifugation. Complete protease inhibitor cocktail (Roche) was added and Aβ40 and Aβ42 were quantified by BAN50/BA27 and BAN50/BC05 ELISAs or 3160/BA27 and 3160/BC05 ELISAs. All measurements were performed in duplicate.

Adenoviral infection of COX1$^{-/-}$ COX-2$^{-/-}$ cells

The adenoviral vector expressing APP695 has been described previously[28]. Primary embryonic fibroblasts derived from mice deficient in COX-1 and COX-2 were infected with 100 plaque-forming units per cell in serum-free medium for 2 h. Medium was changed and cells were then treated with drugs as described above.

APP processing, Aβ clearance and Notch cleavage

Methods and antibodies for analysis of steady-state APP expression, APPs secretion, APP turnover and APP internalization are described in the Supplementary Information. Aβ turnover after NSAID treatment was examined by culturing untransfected CHO cells with conditioned medium obtained from WT-APP-transfected CHO cells. CHO cells were pretreated with sulindac sulphide overnight. Medium was then replaced with the conditioned medium supplemented with increasing concentrations of sulindac sulphide and incubated for another 24 h. Aβ40 and Aβ42 levels in the medium were then analysed by bicine/urea Aβ western blot analysis and ELISA as documented below.

The Myc-tagged, amino-terminal-truncated Notch-1 construct (NΔEMV, in which methionine 1,726 has been mutated to valine to eliminate translation initiation at that site) and the construct containing only the NICD domain have been described[22]. To monitor formation of NICD, the NΔEMV construct was transiently transfected into HEK293 cells. Cells were then treated with drugs for 36 h and subsequently pulse labelled with [^{35}S]-methionine for 30 min and chased for 2 h. Cell lysates were immunoprecipitated with monoclonal antibody 9E10 (Calbiochem) against the Myc-epitope sequence, fractionated by SDS–PAGE and analysed by phosphor imaging.

Mass spectrometry

Secreted Aβ peptides were analysed by IPMS as described[29]. One millilitre of conditioned medium was immunoprecipitated with monoclonal antibody 4G8 (Senetek) and molecular masses and concentrations of Aβ peptides were measured with an ultraviolet-laser desorption/ionization time-of-flight mass spectrometer. To compare the concentrations of individual Aβ species in conditioned medium, synthetic Aβ(12–28) peptide (Sigma) was added to the samples as an internal standard, and relative peak heights were calculated.

Western blotting of Aβ peptides

Bicine/urea Aβ western blot analysis was performed as described[23]. One millilitre of conditioned medium was immunoprecipitated with APP monoclonal antibody 26D6 recognizing amino acids 1–12 of the Aβ sequence[26]. Samples were separated on bicine/urea gels, transferred to nitrocellulose membranes and immunoblotted with 26D6 antibody. Standard Aβ40, Aβ42 and Aβ38 peptides (Sigma) were separated on the same gel for identification of the corresponding Aβ species. Representative radiograms are shown.

Ibuprofen treatment of Tg2576 transgenic mice

Female Tg2576 mice overexpressing APP695 containing the 'Swedish' mutation were treated at 3 months old, when they show high levels of soluble Aβ in brain but no signs of Aβ deposition[30]. NSAIDs were dissolved in DMSO, mixed with a sucrose drink (Kool-Aid) and fed orally to the animals. Controls were administered Kool-Aid with DMSO. The total amount of ibuprofen or naproxen delivered was 50 mg kg^{-1} d^{-1}. This daily dose was divided equally and administered every 4 h for 3 d. Two hours after the final dose, animals were killed and SDS-soluble Aβ40 and Aβ42 were analysed by ELISA as described previously[30].

Received 19 July; accepted 13 September 2001.

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Acknowledgements

We thank X. Zhang and D. A. Young for fibroblasts deficient in COX-1 and COX-2; R. Kopan for Notch plasmids; M. Kounnas for 26D6 antibody; P. Sims, D. Galasko and C. Eckman for discussions; and Takeda industries for the BAN50, BA27 and BC05 antibodies. We also thank P. Needleman for his scientific input. This work was supported by National Institutes of Health grants (E.H.K., T.E.G., R.W.), a Beeson Award (T.E.G.), an Ellison Medical Foundation New Scholars award (T.E.G.), a Robert and Clarice Smith Fellowship (P.D.), a John Douglas French Alzheimer's Foundation Fellowship (M.P.M.), and an Emmy Noether fellowship from the Deutsche Forschungsgemeinschaft (S.W.).

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Gridlock signalling pathway fashions the first embryonic artery

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Arteries and veins are morphologically, functionally and molecularly very different, but how this distinction is established during vasculogenesis is unknown[1,2]. Here we show, by lineage tracking in zebrafish embryos, that angioblast precursors for the trunk artery and vein are spatially mixed in the lateral posterior mesoderm. Progeny of each angioblast, however, are restricted to one of the vessels. This arterial–venous decision is guided by *gridlock* (*grl*), an artery-restricted gene that is expressed in the lateral posterior mesoderm[3]. Graded reduction of *grl* expression, by mutation or morpholino antisense, progressively ablates regions of the artery, and expands contiguous regions of the vein, preceded by an increase in expression of the venous marker EphB4 receptor (*ephb4*)[2] and diminution of expression of the arterial marker ephrin-B2 (*efnb2*)[2]. *grl* is downstream of *notch*[4], and interference with *notch* signalling, by blocking *Su(H)*[4], similarly reduces the artery and increases the vein. Thus, a *notch–grl* pathway controls assembly of the first embryonic artery, apparently by adjudicating an arterial versus venous cell fate decision.

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